**Università degli Studi di Milano**

GRADUATE SCHOOL OF VETERINARY SCIENCES
FOR ANIMAL HEALTH AND FOOD SAFETY
Director: Prof. Valentino Bontempo

**Doctoral Program in Animal Nutrition and Food Safety**

*Academic Year: 2011-2012*

---

# *In vitro* effects of fumonisin $B_1$ alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation and steroidogenesis

**Cristina Cortinovis**

---

Tutor: **Prof. Francesca Caloni**

Coordinator: **Prof. Giovanni Savoini**

# Index


1. **Foreword** **7**
    1.1. Mycotoxins 7
    1.2. *Fusarium* mycotoxins 7
        *1.2.1. Fumonisins 9*
        *1.2.2. Zearalenone 11*
        *1.2.3. Trichothecenes 14*
        *1.2.4. Worldwide contamination 17*
    1.3. Effects of *Fusarium* mycotoxins on ovarian function 20
        *1.3.1. Fumonisins 20*
        *1.3.2. Zearalenone 20*
        *1.3.3. Trichothecenes 23*
    1.4. References 25

2. **Objectives** **39**

3. **Effects of fumonisin $B_1$ alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation** **43**
    3.1. Abstract 43
    3.2. Introduction 43
    3.3. Materials and methods 45
        *3.3.1. Reagents and Hormones 45*
        *3.3.2. Cell Culture 45*
        *3.3.3. Determination of granulosa cell numbers 47*
        *3.3.4. Experimental design 48*
        *3.3.5. Statistical analysis 49*
    3.4. Results 49
        *3.4.1. Experiment 1: effects of $FB_1$ on granulosa cell proliferation in the presence of FSH plus IGF-I or FGF9 or both. 49*
        *3.4.2. Experiment 2: effects of DON and a-ZOL with and without $FB_1$ on FSH plus IGF-I-induced granulosa cell proliferation 50*
        *3.4.3. Experiment 3: effect of $FB_1$ on serum-induced granulosa cell proliferation 51*
    3.5. Discussion 52
    3.6. References 54

4. **Effects of fumonisin $B_1$ alone and combined with deoxynivalenol or zearalenone on steroid production by porcine granulosa cells** **65**

4.1. Abstract 65

4.2. Introduction 66

4.3. Materials and methods 67

*4.3.1. Reagents and hormones 67*

*4.3.2. Cell culture 67*

*4.3.3. Determination of steroid concentrations 70*

*4.3.4. RNA analysis 71*

*4.3.5. Experimental design 73*

*4.3.6. Statistical analysis 73*

4.4. Results 74

*4.4.1. Experiment 1: dose-response of $FB_1$ on steroid production in the presence of FSH with or without IGF-I 74*

*4.4.2. Experiment 2: interaction between $FB_1$ and DON or a-ZOL on FSH plus IGF-I-induced steroid production 74*

*4.4.3. Experiment 3: $FB_1$ effects on granulosa cell CYP11A1 and CYP19A1 mRNA 77*

*4.4.4. Experiment 4: effect of simvastatin on the stimulatory effect of $FB_1$ on FSH plus IGF-I-induced progesterone production 78*

4.5. Discussion 79

4.6. References 82

5. **General discussion** **91**

5.1. References 94

6. **Summary** **101**

7. **Acknowledgements** **105**

# CHAPTER 1

# Foreword

# 1. Foreword

## 1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by the mycelial structure of filamentous fungi, or more specifically, the moulds. Their global occurrence is considered to be a major risk affecting human and animal health as it is estimated that 25% of the world's crop production is contaminated to some extent by mycotoxins (Fink-Gremmels, 1999). The most frequent toxigenic fungi are *Fusarium, Aspergillus* and *Penicillium* species (Creppy, 2002). Mycotoxin contamination may occur in the field before harvest, during harvesting, or during storage and processing. Environmental factors such as substrate composition, humidity and temperature govern the mycotoxin production and thus the degree of contamination of feed and food commodities (Fink-Gremmels, 1999). Mycotoxin contamination can increase in extreme environmental conditions such as drought, excessive precipitation, or sudden frost (Newman and Raymond, 2005). According to their various chemical structure, mycotoxins have a wide spectrum of toxicological effects. The nature and intensity of these effects is related to the dose levels and duration of exposure (Fink-Gremmels, 1999). Mycotoxin exposure via feed may result in acute intoxication. However, the effects and syndromes arising from chronic intake of mycotoxins are likely to be more important in practical situations. Much of concern is about chronic exposure to low doses that may even remain undetected but may result in reduced feed intake, feed refusal, poor feed conversion, diminished body weight gain, increased susceptibility to infections (due to immune-suppression), and reduced reproductive capacities (Fink-Gremmels and Malekinejad, 2007; Morgavi and Riley, 2007; Pestka, 2007; Voss et al., 2007) which leads to economic losses (Binder et al., 2007).

## 1.2. *Fusarium* mycotoxins

Among the genera of fungi most likely to affect animal health and production, *Fusarium* species are particularly significant because they are ubiquitous in nature and produce a wide range of mycotoxins of diverse structure and chemistry (Glenn, 2007; Morgavi and Riley, 2007) (Table 1.1). The *Fusarium* fungi are probably the most prevalent toxin-producing fungi of the northern hemisphere and are commonly found on cereals grown in the temperate regions of America, Europe and Asia (Creppy, 2002).

**Table 1.1.** *Fusarium* species, primary agronomic hosts, endemic regions, and major mycotoxins known to be produced (Glenn, 2007).

| ***Fusarium* species** | **Hosts of primary concern** | **Endemic geographical regions** | **Mycotoxins** [a] |
|---|---|---|---|
| *F. avenaceum* | Corn, wheat, barley, oats | Worldwide | MON, BEA, FUS |
| *F. crookwellense* | Wheat, barley, oats | Worldwide | NIV, ZEA, FUS |
| *F. culmorum* | Corn, wheat, barley, oats | Worldwide | DON, ZEA, NIV, FUS |
| *F. fujikuroi* | Rice | Worldwide | GB, MON, BEA, FB[b] |
| *F. globosum* | Corn | Africa | FB, BEA, FP[b] |
| *F. graminearum* | Corn, wheat, barley, oats | Worldwide | DON, ZEA, NIV, FUS |
| *F. kyushuense* | Wheat | Japan | NIV, T2, DAS |
| *F. langsethiae* | Wheat, barley, oats | Europe | DAS, T2, HT2, BEA[b] |
| *F. napiforme* | Millet, sorghum | Africa, Argentina | FB, MON |
| *F. nygamai* | Sorghum | Africa, Australia | FB, MON, BEA |
| *F. poae* | Wheat, barley, oats | Worldwide | DAS, NIV, BEA, FUS, T2, HT2 |
| *F. proliferatum* | Corn | Worldwide | FB, MON, BEA, FP |
| *F. pseudoanthophilum* | Corn | Africa | BEA |
| *F. pseudograminearum* | Wheat, barley, oats | Africa, Australia, North America | DON, ZEA |
| *F. pseudonygamai* | Millet | Africa | MON, FP |
| *F. sporotrichioides* | Wheat, barley, oats | Worldwide | T2, HT2, DAS, BEA, FUS |
| *F. subglutinans* | Corn | Worldwide | MON, BEA, FP |
| *F. thapsinum* | Sorghum | Worldwide | MON |
| *F. verticillioides* | Corn | Worldwide | FB, FUS, MON[b] |

[a] BEA, beauvericin; DAS, diacetoxyscirpenol; DON, deoxynivalenol; FB, fumonisin $B_1$, $B_2$, and $B_3$; FP, fusaproliferin; FUS, fusarin C; GB, gibberellins; HT2, HT-2 toxin; MON, moniliformin; NIV, nivalenol or its acetylated derivatives; T2, T-2 toxin; ZEA, zearalenone.

[b] Production of this mycotoxin is rare among strains of the species.

A wet growing season followed by cool weather increases the likelihood of *Fusarium* species and its mycotoxins being present in grains (Larsen et al., 2004). The high moisture level encourages fungal growth in grain whilst cool temperatures can increase the production of mycotoxins. *Fusarium* species require a moisture content of 25% (Newman and Raymond, 2005) and toxinogenesis is strictly influenced by temperature and water activity ($a_w$) (Sweeney and Dobson, 1998).
*Fusarium* mycotoxins are endowed with both acute and chronic aspects of toxicity and have shown to cause a broad variety of toxic effects in animals The effects observed are often related to dose levels and duration of exposure. Outbreaks of *Fusarium* mycotoxicoses occurred naturally in Europe, Asia, New Zealand and South America. Furthermore, the risk of continuing exposure has not diminished in spite of enhanced awareness of the debilitating effects of these mycotoxins.
Chronic intake of *Fusarium* mycotoxins represents a continuing hazard and is reported on a regular and more widespread basis due to continuing worldwide contamination of cereal grains and animal feed (D'Mello et al., 1999). From a global perspective the main mycotoxin classes of concern produced by *Fusarium* species include trichothecenes such as deoxynivalenol (DON) and T-2 toxin, fumonisins and zearalenone (ZEA) (Glenn, 2007). Exposure to these mycotoxins has been positively linked with a number of specific syndromes in farm livestock (D'Mello et al., 1999; Morgavi and Riley, 2007). These include feed refusal, emesis and anorexia (DON); oral and gastro-intestinal lesions (T-2 toxin); reproductive dysfunction (ZEA); equine leukoencephalomalacia and porcine pulmonary edema (fumonisins). In addition, duodenitis/ proximal jejunitis and acute mortality syndrome have tentatively been linked with particular *Fusarium* mycotoxins (D'Mello et al., 1999).

### *1.2.1. Fumonisins*

Fumonisins are mycotoxins produced by various moulds of the genus *Fusarium* primarily by *Fusarium verticillioides* (Figure 1.1) and *Fusarium proliferatum,* although other fungal species including *F. napiforme* and *F. nygamai* are also able to produce fumonisins (Glenn, 2007) (Table 1.1). Fumonisins occur particularly in corn and corn-based products and co-occurrence with other *Fusarium* mycotoxins, such as ZEA and DON, is regularly observed (EFSA, 2005). Fumonisins were first isolated from cultures of *Fusarium verticillioides* (formerly *Fusarium moniliforme*) strain MRC 826 at the South African Medical Research Council by Gelderblom et al. (1988) and their structure was elucidated by Bezuidenhout et al. (1988). Since then, twenty-eight fumonisins have been isolated and grouped into four series known as A, B, C and P (Yazar and Omurtag, 2008). The most important

as natural contaminants of cereals are the B series (fumonisins $B_1$, $B_2$ and $B_3$). Fumonisin $B_1$ ($FB_1$), fumonisin $B_2$ ($FB_2$) and fumonisin $B_3$ ($FB_3$) have been shown to occur naturally at significant levels in corn and a variety of corn-based animal feed and human foodstuffs in several countries throughout the world (Sydenham et al., 1991). $FB_1$ (Figure 1.2) is the most significant in terms of toxicity and occurrence (EFSA, 2005). $FB_1$ concentrations usually exceed those of $FB_2$ and $FB_3$ by about three or more times for $FB_2$ and by 12 times for $FB_3$ (Sydenham et al., 1991).

$FB_1$ has a species-specific toxicity in domestic animals as it is the causative agent of leukoencephalomalacia in horses (Marasas et al., 1988; Kellerman et al., 1990), pulmonary edema syndrome in pigs (Harrison et al., 1990; Osweiler et al., 1992; Colvin et al., 1993), hepatocarcinoma in rats (Gelderblom et al., 1991) and mice (Howard et al., 2001a), nephrocarcinoma in rats (Howard et al., 2001b), and nephropathy in rats (Voss et al., 1993) and rabbits (Gumbrecht et al., 1995). Additionally $FB_1$ showed a mild to fatal liver toxicity in many species including pigs, horses, cattle, rabbits and primates (Smith, 2012). $FB_1$ is at present considered to be a possible carcinogen for humans and is classified by the International Agency for Research on Cancer as class 2B (IARC, 2003).

The mechanism of toxicity for $FB_1$ is complex and may involve several molecular sites. $FB_1$ bears a remarkable structural resemblance to the long-chain (sphingoid) base backbones of sphingolipids and thus impairs sphingolipid biosynthesis by specifically inhibiting sphingosine N-acyltransferase (ceramide synthase) (Wang et al., 1991; Merrill et al., 2001; Voss et al., 2007). The inhibition of ceramide synthase leads to the accumulation of sphingoid bases (sphinganine and, to a lesser degree, sphingosine) and to the depletion of complex sphingolipids, which interfere with the function of some membrane proteins (Wang et al., 1991; Merrill et al., 2001; Voss et al., 2007). Because sphingolipids are involved in diverse aspects of cell regulation, disruption of the sphingolipid metabolism may underlie many of the mechanisms for the toxicity and carcinogenicity of fumonisins (Wang et al., 1992; Smith et al., 2002; Marasas et al., 2004).

The European Commission has established guidance levels for fumonisins ($FB_1$ + $FB_2$) in animal feed that vary by species, reflecting their relative sensitivities to fumonisins. These guidance values, concerning complementary and complete feeding stuffs, are 5 mg/kg for horses, rabbit, pigs and pet animals, 10 mg/kg for fish, 20 mg/kg for poultry, calves (<4 months) and lambs and of 50 mg/kg for adult ruminants (>4 months) and mink (EC, 2006).

**Figure 1.1.** Conidiophores and conidia of the fungus *Fusarium verticillioides*.

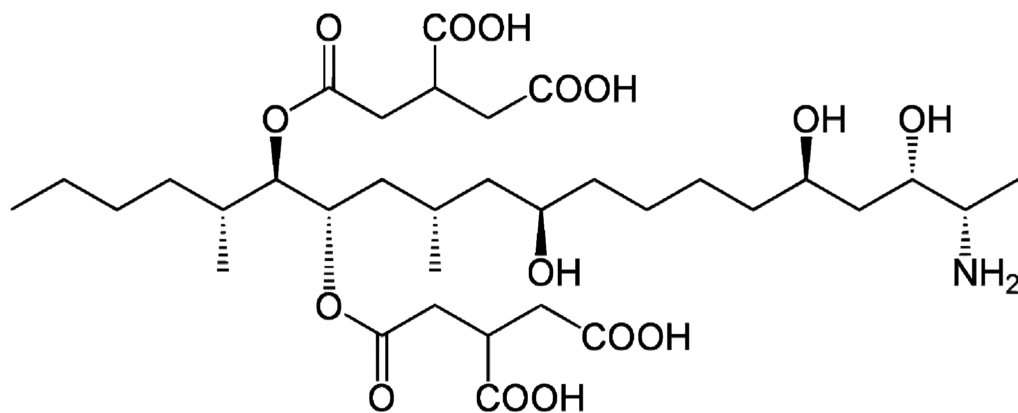



**Figure 1.2.** Chemical structure of fumonisin $B_1$ ($FB_1$).

## *1.2.2. Zearalenone*

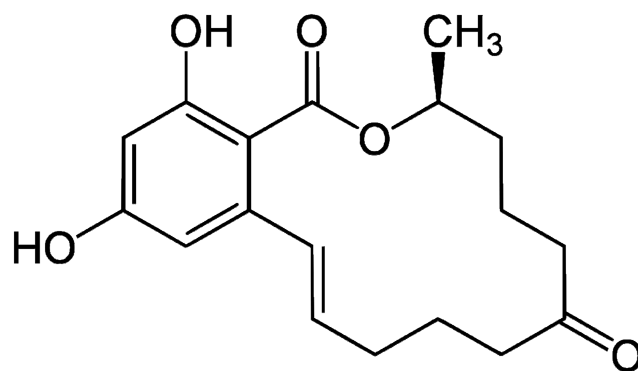
Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin biosynthesized through a polyketide pathway by a variety of *Fusarium* fungi (Zinedine et al., 2007) including *F. graminearum*, *F. culmorum*, *F. pseudograminearum* and *F.*

*crookwellense* (Table 1.1). These species are known to infest primarily wheat, barley, oats and corn (Glenn, 2007). ZEA may co-exist with DON, as the same fungi, *F. graminearum, F. culmorum or F. pseudograminearum*, may produce both compounds (Table 1.1).

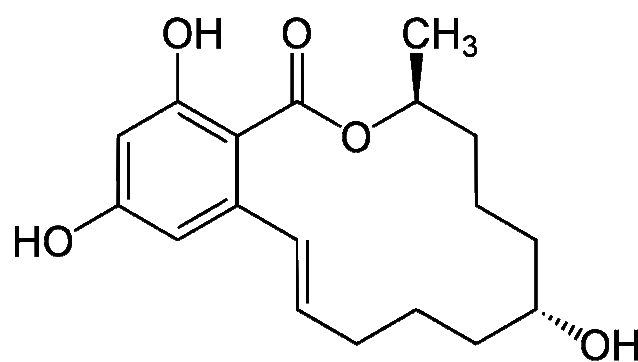
ZEA (Figure 1.3) is a resorcyclic acid lactone, chemically described as 6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone (Zinedine et al., 2007). This structure resembles many characteristics of steroid hormones and allows ZEA to bind to both types of estrogen receptors, estrogen receptor α (ESR1) and estrogen receptor β (ESR2), where it acts as an agonist and partial antagonist to estradiol (Malekinejad et al., 2007). ZEA has been found to induce estrogenic effects, often reported as hyperestrogenism, in all laboratory animal species tested, as well as in farm animals, particularly in pigs. ZEA is rapidly absorbed after oral administration and its uptake is estimated to reach 80-85% (Minervini and Dell'Aquila, 2008). Species differences in the susceptibility to ZEA exposure have been associated with differences in the hepatic and extrahepatic metabolism of ZEA that are catalyzed by hydroxysteroid hydrogenases (Malekinejad et al., 2007). ZEA is reduced primarily to two isomeric metabolites, alpha-zearalenol (α-ZOL) and beta-zearalenol (β-ZOL) (Figure 1.3). Different lines of evidence have indicated that α-ZOL has a higher estrogenic potency compared with the parent ZEA, whereas β-ZOL has a lower potency (Malekinejad et al., 2005). The species-specific sensitivity observed in clinical trials and in field studies correlates with the rate of bioconversion into α-ZOL and identified the pig as the most sensitive farm animal species (Malekinejad et al., 2005).

Common clinical signs in premature gilts include reddening and swelling of the vulva, enlarged nipples, and an enlarged uterus, whereas in cycling sows fertility is impaired (Malekinejad et al., 2007; Minervini and Dell'Aquila, 2008). During pregnancy, ZEA reduces embryonic survival and sometimes decreases fetal weight. In boars depressed serum testosterone, reduced testis weights and spermatogenesis, feminization and suppressing libido have been observed (D'Mello et al., 1999; Malekinejad et al., 2007)

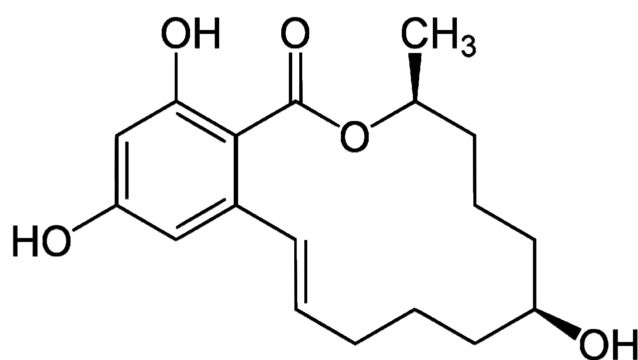
The European Commission has recommended guidance values for ZEA in products intended for animal feed. The guidance values for cereal/cereal products and corn by-products (12% moisture content) are 2 and 3 mg/kg, respectively (EC, 2006). The guidance values for complementary and complete feedingstuffs for piglets/young sows, older sows/fattening pigs, and calves/dairy cattle/sheep/goats are 0.1, 0.25 and 0.5 mg/kg, respectively (EC, 2006).




zearalenone




α-zearalenol




β-zearalenol

**Figure 1.3.** Chemical structure of zearalenone (ZEA) and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL).

### *1.2.3. Trichothecenes*

The trichothecenes are a family of closely related sesquiterpenoids subdivided into four basic groups (A, B, C and D) according to their chemical structures, with type A and type B representing the most important members. The main sources of trichothecenes are contaminated cereal grains, such as corn, wheat, rye, barley and oats. The type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpetol (DAS) while type B include DON and its 3-acetyl and 15-acetyl derivates, nivalenol (NIV) and fusarenon X. It is generally accepted that among the trichothecenes, type A members are more toxic than those in the type B category (Placinta et al., 1999). All trichothecenes share a tricyclic nucleus that contains an epoxide at C-12 and C-13, which is considered essential for toxicity (Mostrom and Raisbeck, 2012).
Trichotecenes have multiple effects on eukaryotic cells, including inhibition of protein, RNA and DNA synthesis, alteration of membrane structure and mitochondrial function, stimulation of lipid peroxidation, induction of programmed cell death or apoptosis and activation of cytokines and chemokines (Mostrom and Raisbeck, 2012). It is believed that the primary effects of trichothecenes is inhibition of protein synthesis as all of the other reported effects might be secondary to decrease protein synthesis (Rocha et al., 2005; Mostrom and Raisbeck, 2012). Trichothecenes bind readily to eukaryotic ribosomes, in particular to the 60S ribosomal subunits, and interfere with peptidyl trasferase activity (Ueno, 1983).
In general, these mycotoxins are capable of producing a variety of toxicoses in animals. Acute exposure of experimental animals to high doses of trichothecenes induces diarrhea, vomiting, leukocytosis, and gastrointestinal hemorrhage. At very high doses, these effects can be accompanied by circulatory shock, reduced cardiac output, and ultimately death (Pestka and Smolinski, 2005). However, under modern agriculture practice, more often chronic exposure to low doses producing anorexia, reduced weight gain, altered nutritional efficiency, and immunotoxicity is observed (Pestka and Smolinski, 2005). Decreased resistance to a number of infectious organisms, such as *Salmonella*, *Mycobacterium*, *Listeria*, *Candida*, *Cryptococcus* and herpes virus, has been documented (Otokawa, 1983; Venturini et al., 1996).

<u>DON</u>
DON is the most commonly encountered thrichotecene relative to frequency and concentration in wheat, corn and barley throughout the world (Rotter et al., 1996; Pestka and Smolinski, 2005). Co-occurrence with other *Fusarium* mycotoxins, including other trichothecenes, ZEA and fumonisins, is regularly observed (EFSA, 2004). DON was first characterized and named following its

isolation from *Fusarium*-infected barley in Japan (Morooka et al., 1972; Yoshizawa and Morooka, 1973). Nearly concurrently, Vesonder et al. (1973) isolated the same compound from *Fusarium*-infected corn in the United States and named it vomitoxin because of its emetic effects in pigs. DON is produced primarily by *Fusarium graminearum* and *Fusarium culmorum* which cause ear rot in corn and head blight in wheat. These two species have two different optimum temperatures for growth (25 ºC and 21 ºC, respectively) and this probably influences their geographical distribution, with *Fusarium graminearum* predominantly occurring in warmer climates.
Structurally, DON (Figure 1.4) is a polar organic compound, which belong to the type B trichothecenes and its chemical name is 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on (Nagy et al., 2005). In its molecule it contains 3 free hydroxy groups (-OH), which are associated with its toxicity (Sobrova et al., 2010).
Although DON is not considered to be acutely toxic to farm animals, it is considered to be a major cause of economic losses due to reduced performance (Morgavi and Riley, 2007). Chronic exposure of farm animals to DON is a continuing hazard in Canada, the USA and continental Europe (D'Mello et al., 1999). The most common effects of prolonged dietary exposure to DON are depressed feed intake, decreased weight gain, feed refusal and impaired immune function with species differences being apparent (D'Mello et al., 1999; Morgavi and Riley, 2007). All animal species evaluated to date are susceptible to DON according to the rank order of pigs > mice > rats > poultry ≈ ruminants. Differences in metabolism, absorption, distribution, and elimination of DON among animal species might account for this differential sensitivity (Pestka and Smolinski, 2005).

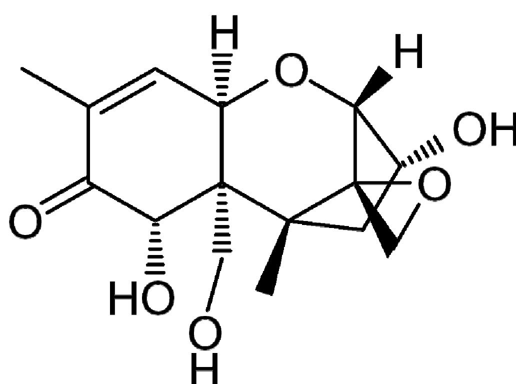



**Figure 1.4.** Chemical structure of deoxynivalenol (DON).

The European Commission (EC) has published guidance levels for DON in products intended for animal feed. These guidance values are 8 mg/kg for

cereals and cereal products with the exception of corn by-products (12 mg/kg) and 5 mg/kg for complementary and complete feeding stuffs with the exception of feeding stuffs for pigs (0.9 mg/kg), calves (<4 months) and lambs (2 mg/kg) (EC, 2006).

T-2 toxin

T-2 toxin (Figure 1.5) is a type-A trichothecene mycotoxin produced by different *Fusarium* species, including *F. sporotrichioides, F. poae*, *F. kyushuense* and *F. langsethiae* (Table 1.1). The principle fungus responsible for the production is *F. sporotrichioides* (Richard, 2007). Some strains of this fungus also produce some closely related mycotoxins (HT-2 toxin and diacetoxyscirpenol) belonging to the same chemical class (Richard, 2007). Corn, wheat, barley, oats, rice, rye and other crops have been reported to contain T-2 toxin (Richard, 2007). The concentration of T-2 toxin in grains is generally lower than the concentrations of DON (Fink-Gremmels, 1999).

This toxin was responsible for the "moldy corn toxicosis" occurring in livestock in North America and alimentary toxic aleukia, a human disease that occurred when several thousand people in Russia consumed cereals that had over-wintered in the fields (Cheeke and Shull, 1985). T-2 toxin is considered the most toxic trichothecene and was the first mycotoxin investigated from this group for its toxicological properties as it may cause severe acute intoxication. T-2 toxin was found to be highly cytotoxic, causing lesions of the mucosa of the gastrointestinal tract, and to lead to a pronounced pancytopenia and immuno-soppression inhibiting protein synthesis and impairing the maturation of hemopoetic cells in bone marrow (Fink-Gremmels, 1999). T-2 toxin, *in vivo*, is readily metabolized to HT-2 toxin. Little direct information is available on the toxicity of HT-2 toxin alone, however, the few comparative data available on T-2 and HT-2 toxins indicate that they induce toxic effects with similar potency (EFSA, 2011).

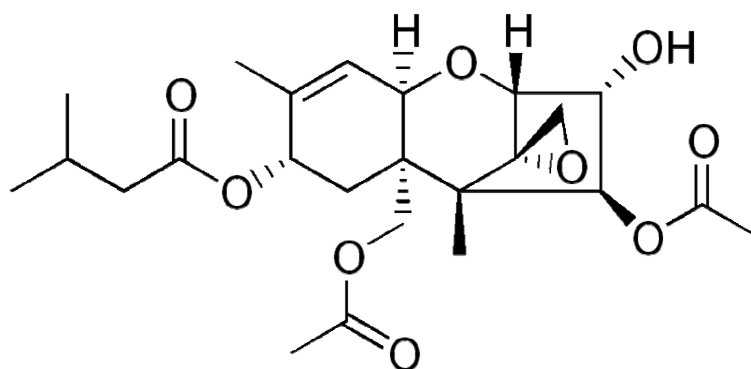



**Figure 1.5.** Chemical structure of T-2 toxin.

Since data about the occurrence and toxic effects of T-2 and HT-2 toxin are scarce, there are no specific regulations or recommendations of the European Commission about the maximal concentrations in products intended for animal feed.

### *1.2.4. Worldwide contamination*

The surveillance of grain and animal feed for the occurrence of *Fusarium* mycotoxins continues to attract worldwide attention and has been the subject of extensive investigations over recent years. Mycotoxins from *Fusarium* species have traditionally been associated with temperate cereals, since these fungi require lower temperatures for growth and mycotoxin production than the aflatoxigenic *Aspergillus* species (Placinta et al., 1999). However, extensive data exists to evidence the global contamination of cereal grains with *Fusarium* mycotoxins particularly trichothecenes, fumonisins and ZEA (Placinta et al., 1999; Binder et al., 2007; Rodrigues and Naehrer, 2012). Trade in these commodities may, therefore, contribute to the worldwide dispersal of mycotoxins (Placinta et al., 1999). Binder et al. (2007) provided an international exhaustive survey spanning several regions of the world of the high global prevalence of *Fusarium* mycotoxins in cereal grains and animal feed. More recent data confirms the widespread distribution of these mycotoxins particularly with respect to DON (Boutigny et al., 2012; Cortinovis et al., 2012 Grajewski et al., 2012) and fumonisins (Boutigny et al., 2012; Garrido et al., 2012). In many of the positive samples, the concentration of toxins was high enough to cause overt clinical signs of toxicoses. In Table 1.2 and Table 1.3 the results on the occurrence of fumonisins, DON and ZEA reported in a three-year study carried out by Rodrigues and Naehrer (2012) are summarized. Between January 2009 and December 2011 corn, soybean/soybean meal, wheat and finished feed samples sourced directly at animal farms or animal feed production sites in the Americas, Europe, Asia and Oceania were analyzed. Fumonisins, DON and ZEA were present in 64%, 59% and 45% of analyzed samples, respectively.
Finally, it has to be underlined that co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is a common feature of several surveys, raising the possibility of toxicological interactions (Girgis and Smith, 2010).

**Table 1.2.** Fumonisins (FUM), deoxynivalenol (DON) and zearalenone (ZEA) occurrence in samples of corn, soybean meal, wheat/wheat bran and finished feed surveyed in North and South America and in Northern, Central and Southern Europe (no corn/soybean meal samples were sourced in Northern Europe; Rodrigues and Naehrer, 2012).

| | North America | | | South America | | | Northern Europe | | | Central Europe | | | Southern Europe | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ZEA | DON | FUM | ZEA | DON | FUM | ZEA | DON | FUM | ZEA | DON | FUM | ZEA | DON | FUM |
| **Corn** | | | | | | | | | | | | | | | |
| total number | 395 | 390 | 466 | 321 | 322 | 807 | - | - | - | 379 | 535 | 30 | 52 | 59 | 48 |
| % positive | 29 | 79 | 39 | 43 | 17 | 92 | - | - | - | 39 | 72 | 60 | 21 | 47 | 90 |
| mean (mg/kg)[a] | 0.3 | 1.1 | 1.4 | 0.2 | 0.2 | 3.2 | - | - | - | 0.1 | 1.4 | 2.2 | 0.3 | 1.0 | 2.3 |
| max (mg/kg)[b] | 4.8 | 24.9 | 22.9 | 1.8 | 0.9 | 53.7 | - | - | - | 0.8 | 26.1 | 7.7 | 1.5 | 3.9 | 11.1 |
| **Soybean meal** | | | | | | | | | | | | | | | |
| total number | 50 | 45 | 46 | 53 | 55 | 60 | - | - | - | 31 | 43 | 2 | 23 | 25 | 21 |
| % positive | 10 | 18 | 0 | 34 | 29 | 5 | - | - | - | 6 | 21 | 0 | 0 | 24 | 29 |
| mean (mg/kg) | 0.1 | 1.0 | - | 0.1 | 0.2 | 0.2 | - | - | - | 0.0 | 0.5 | - | - | 0.4 | 1.0 |
| max (mg/kg) | 0.1 | 5.5 | - | 0.8 | 0.4 | 0.3 | - | - | - | 0.1 | 0.7 | - | - | 0.9 | 5.1 |
| **Wheat/wheat bran** | | | | | | | | | | | | | | | |
| total number | 16 | 25 | 7 | 32 | 17 | 40 | 71 | 71 | 1 | 256 | 436 | 9 | 17 | 24 | 10 |
| % positive | 13 | 76 | 0 | 47 | 53 | 5 | 15 | 55 | 0 | 12 | 55 | 33 | 0 | 38 | 30 |
| mean (mg/kg) | 0.3 | 1.0 | - | 0.1 | 0.9 | 1.4 | 0.1 | 1.1 | - | 0.1 | 1.5 | 0.3 | - | 1.2 | 0.4 |
| max (mg/kg) | 0.5 | 7.0 | - | 0.4 | 2.5 | 1.7 | 0.2 | 7.3 | - | 0.3 | 49.0 | 0.5 | - | 3.5 | 0.9 |
| **Finished feed** | | | | | | | | | | | | | | | |
| total number | 42 | 55 | 32 | 119 | 130 | 224 | 27 | 27 | 1 | 489 | 579 | 65 | 72 | 104 | 48 |
| % positive | 52 | 65 | 47 | 57 | 13 | 94 | 37 | 74 | 0 | 48 | 67 | 40 | 18 | 37 | 75 |
| mean (mg/kg) | 0.3 | 1.7 | 2.4 | 0.2 | 0.3 | 1.7 | 0.1 | 0.6 | - | 0.1 | 0.8 | 0.3 | 0.1 | 0.4 | 2.0 |
| max (mg/kg) | 1.7 | 6.1 | 11.4 | 3.6 | 0.8 | 10.4 | 0.3 | 1.9 | - | 1.0 | 25.8 | 2.3 | 0.2 | 1.3 | 7.0 |

[a] Mean of positive samples.
[b] Maximum of positive samples.

**Table 1.3.** Fumonisins (FUM), deoxynivalenol (DON) and zearalenone (ZEA) occurrence in samples of corn, soybean meal, wheat/wheat bran and finished feed surveyed in North, South-East and South Asia and in Oceania (no wheat/wheat bran samples were sourced in South Asia; Rodrigues and Naehrer, 2012).

| | North Asia | | | South-East Asia | | | South Asia | | | Oceania | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ZEA | DON | FUM | ZEA | DON | FUM | ZEA | DON | FUM | ZEA | DON | FUM |
| **Corn** | | | | | | | | | | | | |
| total number | 470 | 477 | 443 | 319 | 218 | 326 | 108 | 106 | 108 | 11 | 11 | 11 |
| % positive | 67 | 92 | 75 | 20 | 45 | 83 | 9 | 22 | 74 | 27 | 27 | 64 |
| mean (mg/kg)[a] | 0.4 | 1.2 | 2.8 | 0.3 | 0.3 | 1.6 | 0.3 | 0.3 | 0.8 | 0.6 | 0.2 | 2.8 |
| max (mg/kg)[b] | 7.4 | 15.1 | 23.5 | 2.6 | 4.8 | 19.3 | 1.1 | 1.2 | 6.2 | 1.3 | 0.2 | 5.4 |
| **Soybean meal** | | | | | | | | | | | | |
| total number | 34 | 37 | 35 | 105 | 105 | 109 | 16 | 16 | 16 | 3 | 3 | 3 |
| % positive | 35 | 38 | 6 | 15 | 18 | 4 | 0 | 31 | 0 | 33 | 33 | 0 |
| mean (mg/kg) | 0.1 | 0.1 | 0.3 | 0.0 | 0.2 | 0.3 | - | 0.2 | - | 0.0 | 0.2 | - |
| max (mg/kg) | 0.4 | 0.3 | 0.3 | 0.1 | 1.0 | 0.4 | - | 0.3 | - | 0.0 | 0.2 | - |
| **Wheat/wheat bran** | | | | | | | | | | | | |
| total number | 72 | 75 | 73 | 40 | 40 | 40 | - | - | - | 115 | 109 | 109 |
| % positive | 42 | 87 | 11 | 40 | 65 | 5 | - | - | - | 28 | 48 | 12 |
| mean (mg/kg) | 0.1 | 0.9 | 0.4 | 0.5 | 2.3 | 0.2 | - | - | - | 1.5 | 5.0 | 0.3 |
| max (mg/kg) | 0.5 | 5.3 | 0.9 | 6.6 | 41.4 | 0.3 | - | - | - | 23.3 | 49.3 | 1.2 |
| **Finished feed** | | | | | | | | | | | | |
| total number | 661 | 671 | 604 | 454 | 447 | 465 | 120 | 111 | 123 | 86 | 86 | 74 |
| % positive | 79 | 89 | 67 | 66 | 35 | 71 | 49 | 22 | 71 | 26 | 34 | 14 |
| mean (mg/kg) | 0.3 | 0.8 | 1.5 | 0.1 | 0.3 | 0.8 | 0.1 | 0.2 | 0.4 | 0.3 | 0.3 | 1.3 |
| max (mg/kg) | 5.8 | 19.1 | 77.5 | 0.3 | 2.7 | 22.7 | 0.2 | 0.6 | 1.5 | 0.9 | 0.7 | 3.2 |

[a] Mean of positive samples.
[b] Maximum of positive samples.

## 1.3. Effects of *Fusarium* mycotoxins on ovarian function

### *1.3.1. Fumonisins*

Though fumonisins were reported to have some deleterious effects on reproduction in rats (Flynn et al., 1996), Syrian hamsters (Floss et al., 1994), and chickens (Javed et al., 1993; Bacon et al., 1995), and to have the potential to impair fertility capacity in boars (Gbore and Egbunikem, 2008; Gbore, 2009), stallions (Minervini et al., 2010) and rabbits (Ewuola and Egbunike, 2010) until now, no study has evaluated direct effects of $FB_1$ on the ovary.

### *1.3.2. Zearalenone*

In cycling sows ZEA causes several reproductive dysfunctions including nymphomania, pseudopregnancy, ovarian atrophy and changes in the endometrium. ZEA causes sterility in sows by inciting a malfunction of the ovary (Mirocha et al., 1977; Minervini and Dell'Aquila, 2008). The oocyte dies in the Graafian follicles and despite signs of estrus, there is no ovulation. ZEA acts similarly to estradiol in inhibiting the release and secretion of follicle stimulating hormone (FSH), thus depressing the maturation of ovarian follicles during the preovulatory stage (Mirocha et al., 1977; Minervini and Dell'Aquila, 2008) The changes induced by ZEA depend on time of administration in relation to estrous cycle as well as on the dose administered (Minervini and Dell'Aquila, 2008).
Chang et al. (1979) reported that concentrations of 25 to 100 mg/kg of 95% purified ZEA fed to multiparous sows after weaning or throughout the gestation period (or both) produced constant estrus, pseudopregnancy, infertility, reduced litter size, malformation and probably fetal resorption. Subsequently, Flowers et al. (1987) observed longer inter-estrous intervals in gilts receiving 20 mg of ZEA on day 6 to 10 or day 11 to 15 of the estrous cycle. Extended cycles were also reported in gilts fed 5 or 10 mg/kg of purified ZEA between day 5 and 20 of the estrous cycle (Edwards et al., 1987). Luteal function was maintained due to a high plasma progesterone level recorded at day 19 to 21 in those gilts that had extended cycles. The persistent corpora lutea spontaneously regressed 30 days after withdrawal of the contaminated feed (Edwards et al., 1987). These results indicated that ZEA may exhibit a luteotrophic property which prolongs the life span of the corpus luteum (Minervini and Dell'Aquila, 2008). In another study, a diet containing 3.61 or 4.33 mg/kg ZEA was fed at a mean daily level of 2 kg/animal to gilts from puberty to mating (Etienne and Jemmali, 1982). In 45% of these gilts ZEA induced a pseudopregnancy state; no estrus was detected within 50 days after puberty and corpora lutea developed at puberty were

maintained (Etienne and Jemmali, 1982). Similar results were obtained by Young and King (1986) in pubertal gilts that consumed 6 or 9 mg/kg of purified ZEA starting the day after first estrus. 88% of the gilts became pseudopregnant as confirmed by plasma progesterone levels and examination of their reproductive tracts (Young and King, 1986). Induced retention of corpora lutea by ZEA was similar to that which occurs when sows are given exogenous estrogen on day 11 to 12 of the estrous cycle (Kidder et al., 1955; Gardner et al., 1963). Young et al. (1990) showed a linear relation between the level of ZEA in mg/kg and the length of anestrus in days. The weaning-to-estrus interval was found to increase when increased dietary ZEA was fed (Young et al., 1990).
More recently *in vitro* studies were carried out to elucidate the interaction of ZEA on the sexual cells of animals. Alm et al. (2002) investigated the influence of different doses of ZEA metabolites, α-ZOL and β-ZOL, on *in vitro* maturation of porcine oocytes. α-ZOL and β-ZOL were found to negatively affect maturation and degeneration rates of porcine oocytes in a dose-dependent manner, but to different extents. Culture of oocytes in the presence of α-ZOL for 48 h up to the concentration of 7.5 μM resulted in a significant decrease in the maturation rate, whereas β-ZOL showed a significant effect only at 30 μM (Alm et al., 2002). α-ZOL had a greater effect than did β-ZOL, probably by means of its greater estrogenicity (Alm et al., 2002). Consistent with these results, ZEA showed to interfere with oocyte progression through meiosis by inducing malformation of the meiotic spindles (Malekinejad et al., 2007). Importantly, ZEA-induced spindle malformations in the oocyte can result in aneuploid embryos (Malekinejad et al., 2007). In a further study, Alm et al. (2006) observed that feeding gilts with wheat naturally contaminated with DON and ZEA negatively influence initial chromatin status of oocyte and oocyte maturation competence *in vitro*. On the contrary, no effect was observed on the expression of the enzyme P450 side-chain cleavage (P450scc) and the enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) in granulosa cells collected from ovaries of gilts fed the contaminated diet (Alm et al., 2006). This indicates that DON and ZEA did not influence the activity of these enzymes involved in progesterone synthesis. These results are not in agreement with previously published *in vitro* studies (Tiemann et al., 2003) which show an apparent influence of ZEA on the expression of P450scc (CYP11A1) and 3β-HSD and therefore reduced progesterone synthesis in cultured porcine granulosa cells. The *in vitro* addition of α-ZOL and β-ZOL at concentrations of 15 and 30 μM inhibited the FSH-stimulated progesterone synthesis (Tiemann et al., 2003). Consistent with these results, in a subsequent *in vitro* study (Ranzenigo et al., 2008) α-ZOL (9.4 μM) was found to decrease abundance of CYP19A1 and CYP11A1 mRNA induced by FSH plus insulin-like growth factor-I (IGF-I). However in the present study α-ZOL primarily increased progesterone

production induced by FSH and IGF-I (Ranzenigo et al., 2008). Estradiol production exhibited a biphasic dose-response to α-ZOL, with 0.094 μM increasing and 9.4 μM inhibiting FSH plus IGF-I-induced estradiol production (Ranzenigo et al., 2008). Recently Zhu et al. (2012) demonstrated that ZEA at high concentrations (30-120 μM) decreases the proliferation of porcine granulosa cells and causes an apoptosis and necrosis in porcine granulosa cells in a dose-dependent manner. The study revealed that ZEA leads to the loss of mitochondrial transmembrane potential of porcine granulosa cells but increases ROS levels of the cells. The authors concluded that ZEA induces an apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9- dependent mitochondrial pathway (Zhu et al., 2012).

Ruminants are significantly less sensitive to ZEA exposure compared to pigs. However, in cows infertility, reduced milk production and hyperestrogenism have been associated with ZEA (D'Mello et al., 1999; Minervini and Dell'Aquila, 2008). When dairy heifers were given daily 250 mg of ZEA (99% purified) over three estrous cycles, the conception rate declined from 87 to 62% (Weaver et al., 1986). Additionally, ZEA from pastures in New Zealand was a recognised cause of infertility in cattle and sheep (Towers and Sprosen, 1993). Minervini et al. (2001) demonstrated *in vitro* a negative effect of ZEA and its derivative, α-ZOL, on meiotic progression of bovine oocytes. Maturation of oocytes to metaphase II was inhibited in oocytes cultured in the presence of 94 μM of ZEA or α-ZOL, with a significant increase in chromatin abnormalities, particularly after the addition of α-ZOL. In the study carried out by Takagi et al. (2008) oocytes were exposed to lower concentration of ZEA and no significant differences in the occurrence of metaphase I and metaphase II were observed between the control group and the group supplemented with <0.31 μM ZEA. A significant decrease in the maturation rates of the oocytes exposed to ZEA 3.1 μM was observed; the maturation of 50% (62/124) of the examined oocytes was arrested in metaphase I (Takagi et al., 2008).

Concerning the equine species, the effects of ZEA have only been demonstrated in a few cases. In cycling mares the effect of 10- day low-dose ZEA exposure on the reproductive parameters was studied (Juhasz et al., 2001). No effect on the length of the interovulatory intervals, luteal and follicular phases was observed in mares fed 7 mg purified ZEA daily starting 10 days after ovulation. ZEA did not influence significantly the plasma progesterone profiles and the follicular activity (growth rate, maximum size of the ovulatory follicles, maximum number and the time of first increase in the number of large follicles) (Juhasz et al., 2001).
In a subsequent study, feeding oats naturally contaminated with ZEA and DON (12 and 1 mg/kg, respectively) had no relevant effects on the release of

reproductive hormones, cycle length and uterine histology in mares (Aurich et al., 2006). However, the authors stated that local ovarian effects of ZEA and DON on follicular growth can not be excluded as the two mycotoxins tended to increase the number of growing follicles during the second half of the cycle. In addition, mares fed the naturally contaminated oats had a high incidence of hemorrhagic corpora lutea and follicular hematomas, which did not occur during control cycles (Aurich et al., 2006).
The *in vitro* exposure of granulosa cells collected from the ovaries of cycling mares with ZEA and its derivatives α-ZOL and β-ZOL induced a simultaneous increase in cell proliferation and an apoptotic process (Minervini et al., 2006). The contemporaneous presence of both processes led the authors to suggest that these mycotoxins could be effective in inducing follicular atresia and these effects may result from both direct interaction with ERs as well as interaction with the enzymes 3-α-(β-)HSD present in the ovary and granulosa cells and involved in the synthesis and metabolism of endogenous steroid hormones (Minervini et al., 2006).

### *1.3.3. Trichothecenes*

DON

The potential of the trichothecenes to act as endocrine disruptors has been the subject of more recent research and continues to be investigated (Ndossi et al., 2012). DON has been implicated in reduced reproductive performance in pigs, due to its ability to impair oocyte maturation and embryo development (Alm et al., 2002, 2006; Malekinejad et al., 2007; Schoevers et al., 2010) and to reduce feed intake (Tiemann and Danicke, 2007).
In the study carried out by Alm et al. (2002), DON was found to have a potent effect on *in vitro* porcine oocyte maturation, significantly decreasing the proportion of oocytes reaching metaphase II at lower concentrations than that of α-ZOL and β-ZOL. In a subsequent study, to evaluate DON toxicity during specific stages of oocyte meiosis, cumulus-oocyte complexes collected from ovaries of cyclic sows were exposed to 0.02, 0.2 and 2 μM DON (Schoevers et al., 2010). Exposure to the highest DON concentration inhibited cumulus expansion and induced cumulus cell death (Schoevers et al., 2010). Absence of cumulus cell expansion and induction of cumulus cell death negatively influence oocyte maturation by altering glutathione levels in the oocyte (Qian et al., 2003). DON at all concentrations reduced metaphase II formation in line with the results obtained by Alm et al. (2002) and led to malformations of the meiotic spindle as previously observed by the same authors (Malekinejad et al., 2007). When oocytes were exposed to 2 μM DON, spindle aberrations occurred at the metaphase I stage, and oocytes maturing in the presence of 0.2 or 0.02 μM DON

exhibited spindle aberrations after reaching the metaphase II stage (Schoevers et al., 2010). Spindle malformation was observed when oocytes were exposed to DON during formation of meiotic spindles at metaphase I and II, but embryo development was also reduced by DON exposure during prophase I. These results indicate that exposure to DON can adversely affect maturation of porcine oocytes by causing abnormalities of the meiotic spindles and by disturbing oocyte cytoplasmic maturation (Schoevers et al., 2010).
Moreover, DON has been shown to have direct dose-dependent effects on porcine granulosa cell proliferation, steroidogenesis and gene expression (Ranzenigo et al., 2008; Medvedove et al., 2011). In the study conducted by Ranzenigo et al. (2008), DON had a biphasic effect on cell growth with 0.034 and 0.34 μM increasing cell numbers and 3.4 μM drastically inhibiting cell numbers. DON at 0.34 μM and 3.4 μM inhibited progesterone and estradiol production induced by FSH plus IGF-I and at 3.4 μM completely blocked the FSH plus IGF-I-induced CYP19A1 and CYP11A1 mRNA abundance (Ranzenigo et al., 2008). On the contrary, Medvedova et al. (2011) observed that DON at the dose of 3.4 μM increased the expression of markers of proliferation (cyclin B1 and PCNA) and stimulated progesterone release in porcine granulosa cells; however this occurred at shorter incubation times (24 h) than in the study carried out by Ranzenigo et al. (2008) (48 h).

T-2 toxin

The effect of oral exposure to low doses of T-2 toxin on the ovarian function was evaluated in ewes and heifers (Huszenicza et al., 2000). The study showed that the peroral T-2 intake can significantly delay the follicle maturation, postpone the subsequent ovulation and may also possibly retard the consecutive luteinisation. As a consequence, animals inseminated during visible signs of standing heat may not conceive. Although the trial did not explain the etiopathogenesis of T-2 toxin upon ovarian functions, it was suggested that the toxin directly deteriorates the granulosa cells and perhaps even the cells of the developing corpus luteum (Huszenicza et al., 2000). On the contrary, low-dose exposure to T-2 toxin in mares had no effect on the length of the interovulatory interval and on the luteal and follicular phases (Juhasz et al., 2001). Similar to DON, in a subsequent *in vitro* study, the potential impact of T-2 toxin on reproductive activity in pigs has been investigated by looking at the effects of this mycotoxin on porcine granulosa cell functions (Caloni et al., 2009). T-2 toxin was found to have potent direct dose-dependent effects on granulosa cell proliferation and steroidogenesis. In details, T-2 toxin strongly inhibited FSH plus IGF-I-induced progesterone and to a greater extent estradiol production as well as cell numbers. It was concluded that these direct ovarian effects could be

one mechanism whereby the presence of T-2 toxin in feedstuffs could impact reproductive performance in swine (Caloni et al., 2009).

## 1.4. References

**Alm H., Brussow K.P., Torner H., Vanselow J., Tomek W., Danicke S., Tiemann U.** (2006). Influence of *Fusarium*-toxin contaminated feed on initial quality and meiotic competence of gilt oocytes. *Reproductive Toxicology, 22: 44-50.*

**Alm H., Greising T., Brüssow K.P., Torner H., Tiemann U.** (2002). The influence of the mycotoxins deoxynivalenol and zearalenol on *in vitro* maturation of pig oocytes and *in vitro* culture of pig zygotes. *Toxicology In Vitro, 16: 643-648.*

**Aurich J.E., Hoppen H.O., Trampler R., Zentek J., Boehm J., Razzazi-Fazeli E., Aurich C.** (2006). Effects of mycotoxins on reproductive function in mares. *Animal Reproduction Science, 94: 238-241.*

**Bacon C.W., Porter J.K., Norred W.P.** (1995). Toxic interaction of fumonisin $B_1$ and fusaric acid measured by injection into fertile chicken egg. *Mycopathologia, 129: 29-35.*

**Bezuidenhout S.C., Gelderblom W.C.A., Gorst-Allman C.P., Horak R.M., Marasas W.F.O., Vleggar R.** (1988). Structure elucidation of fumonisins, mycotoxins from *Fusarium moniliforme. Journal of The Chemical Society, Chemical Communications, 19: 743-745.*

**Binder E.M., Tan L.M., Chin L.J., Handl J., Richard J.** (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal Feed Science and Technology, 137: 265-282.*

**Boutigny A.L., Beukes I., Small I., Zuhlke S., Spiteller M., Van Rensburg B.J., Flett B., Viljoen A.** (2012). Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. *Plant pathology, 61: 522-531.*

**Caloni F., Ranzenigo G., Cremonesi F., Spicer L.J.** (2009). Effects of a trichothecene, T-2 toxin, on proliferation and steroid production by porcine granulosa cells. *Toxicon, 54: 337-344.*

**Chang K., Kurtz H.J., Mirocha C.J.** (1979). Effects of the mycotoxin zearalenone on swine reproduction. *American Journal of Veterinary Research, 40: 1260-1270.*

**Cheeke P.R., Shull L.R.** (1985). Natural Toxicants in Feeds and Poisonous Plants. *AVI Publishing, Westport, CT.*

**Colvin B.M., Cooley A.J., Beaver R.W.** (1993). Fumonisin toxicosis in swine: clinical and pathologic findings. *Journal of Veterinary Diagnostic Investigation, 5: 232-241.*

**Cortinovis C., Battini M., Caloni F.** (2012). Deoxynivalenol and T-2 Toxin in Raw Feeds for Horses. *Journal of Equine Veterinary Science, 32: 72-74.*

**Creppy E.E.** (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127: 19-28.*

**D'Mello J.P.F., Placinta C.M., Macdonald A.M.C.** (1999). *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology, 80: 183-205.*

**Edwards S., Cantley T.C., Rottinghaus G.E., Osweiler G.D., Day B.N.** (1987). The effects of zearalenone on reproduction in swine. I. The relationship between ingested zearalenone dose and anestrus in non-pregnant, sexually mature gilts. *Theriogenology, 28: 43-49.*

**EFSA (European Food Safety Authority)** (2004). Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to deoxynivalenol (DON) as undesirable substance in animal feed. *EFSA Journal, 73: 1-42.*

**EFSA (European Food Safety Authority)** (2005). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to fumonisins as undesirable substances in animal feed. *EFSA Journal, 235: 1-32.*

**EFSA (European Food Safety Authority)** (2011). Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. *EFSA Journal, 9: 2481.*

**Etienne M., Jemmali M.** (1982). Effects of zearalenone (F2) on estrous activity and reproduction in gilts. *Journal of Animal Science, 55: 1-10.*

**European Commission** (2006). Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). *Official Journal of the European Union L, 229: 7-9.*

**Ewuola E.O., Egbunike G.N.** (2010). Effects of dietary fumonisin $B_1$ on the onset of puberty, semen quality, fertility rates and testicular morphology in male rabbits. *Reproduction, 13: 439-445.*

**Fink-Gremmels J.** (1999). Mycotoxins: their implications for human and animal health. *The Veterinary Quarterly, 21: 115-120.*

**Fink-Gremmels J., Malekinejad H.** (2007). Biochemical mechanisms and clinical effects associated with exposure to the mycoestrogen zearalenone. *Animal Feed Science and Technology, 137: 326-341.*

**Floss J.L., Casteel S.W., Johnson G.C., Rottinghaus G.E., Krause G.F.** (1994). Development toxicity of fumonisin in Syrian hamsters. *Mycopathologia, 128: 33-38.*

**Flowers B., Cantley T., Day B.N.** (1987). A comparison of effects of zearalenone and estradiol benzoate on reproductive function during the estrous cycle in gilts. *Journal of Animal Science, 65: 1576-1584.*

**Flynn T.J., Pritchard D., Bradlaw J., Eppley R., Page S.** (1996). *In vitro* embryotoxicity of fumonisin $B_1$ evaluated with cultured postimplantation staged rat embryos. *In Vitro Toxicology, 9: 271-279.*

**Gardner M.L., First N.L., Casida L.E.** (1963). Effect of exogenous estrogens on corpus luteum maintenance in gilts. *Journal of Animal Science, 22: 132-134.*

**Garrido C.E., Hernández Pezzani C., Pacin A.** (2012). Mycotoxins occurrence in Argentina's maize (Zea mays L.), from 1999 to 2010. *Food Control, 2: 660-665.*

**Gbore F.A., Egbunike G.N.** (2008). Testicular and epididymal sperm reserves and sperm production of pubertal boars fed dietary fumonisin $B_1$. *Animal Reproduction Science, 105: 392-397.*

**Gbore F.A.** (2009). Reproductive organ weights and semen quality of pubertal boars fed dietary fumonisin $B_1$. *Animal, 3: 1133-1137.*

**Gelderblom W.C., Jaskiewicz K., Marasas W.F.O., Thiel P.G., Horak R.M., Vlegaar R., Kriek N.P.** (1988). Fumonisins–novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Applied and Environmental Microbiology, 54: 1806-1811.*

**Gelderblom W.C., Kriek N.P., Marasas W.F.O., Thiel P.G.** (1991). Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin $B_1$, in rats. *Carcinogenesis, 12: 1247-1251.*

**Girgis G.N., Smith T.K.** (2010). Comparative aspects of *Fusarium* mycotoxicoses in poultry fed diets containing naturally contaminated grains. *World's Poultry Science Journal, 66: 65-86.*

**Glenn A.E.** (2007). Mycotoxigenic *Fusarium* species in animal feed. *Animal Feed Science and Technology, 137: 213-240.*

**Grajewski J., Błajet-Kosicka A., Twarużek M., Kosicki R.** (2012). Occurrence of mycotoxins in Polish animal feed in years 2006-2009. *Journal of Animal Physiology and Animal Nutrition, 96: 870-877.*

**Gumbrecht L.A., Marcucci A., Weigel R.M., Vesconder R.F., Riley R.T., Snowker J.L., Beasly V.R., Haschek W.** (1995). Effects of intravenous Fumonisin $B_1$ in rabbits: nephrotoxicity and sphingolipid alterations. *Natural Toxins, 3: 395-403.*

**Harrison L.R., Colvin B.M., Greene J.T., Newman L.E., Cole J.R. Jr.** (1990). Pulmonary edema and hydrothorax in swine produced by fumonisin $B_1$, a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostic Investigation, 2: 217-221.*

**Howard P.C., Eppley R.M., Stack M.E., Warbritton A., Voss K.A., Lorentzen R.J., Kovach R.M., Bucci T.J.** (2001a). Fumonisin $B_1$ carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. *Environmental Health Perspectives, 109: 277-282.*

**Howard P.C., Warbritton A., Voss K.A., Lorentzen R.J., Thurman J.D., Kovach R.M., Bucci T.J.** (2001b). Compensatory regeneration as a mechanism

for renal tubule carcinogenesis of fumonisin $B_1$ in F344/N/Nctr BR rats. *Environmental Health Perspectives, 109: 309-314.*

**Huszenicza G., Fekete S., Szigeti G., Kulcsár M., Fébel H., Kellems R.O., Nagy P., Cseh S., Veresegyházy T., Hullár I.** (2000). Ovarian consequences of low dose peroral *Fusarium* (T-2) toxin in a ewe and heifer model. *Theriogenology, 53: 1631-1639.*

**IARC** (2003). Toxins derived from *Fuasrium* moniliforme: Fumonisin $B_1$ and $B_2$ and fusarin C. *Monograph and Evaluation of Carcinogenic Risks to Human, Lyon, France, pp. 445-456.*

**Javed T., Richard J.L., Bennett G.A., Dombrink-Kurtzman M.A., Bunte R.M., Koelkebeck K.W., Côté L.M., Leeper R.W., Buck W.B.** (1993). Embryopathic and embryocidal effects of purified fumonisin $B_1$ or *Fusarium proliferatum* culture material extract on chicken embryos. *Mycopathologia, 123: 185-193.*

**Juhasz J., Nagy P., Kulesar M., Szigeti G., Reiczigel J., Huszenicza G.** (2001). Effect of low-dose zearalenone exposure on luteal function, follicular activity and uterine oedema in cycling mares. *Acta Veterinaria Hungarica, 49: 211-222.*

**Juhasz J., Nagy P., Reiczigel J., Kulcsar M., Szigeti G., Huszenicza G.** (2011). Effect of certain mycotoxins contaminating feedstuff (zearalenone, T-2 toxin) and dexamethasone on the reproductive function of horses. *Magyar Allatorvosok Lapja, 123: 475-482.*

**Kellerman T.S., Marasas W.F.O., Thiel P.G., Gelderblom W.C., Cawood M., Coetzer J.A.** (1990). Leukoencephalomalacia in two horses induced by oral dosing of fumonisin $B_1$. *Onderstepoort Journal of Veterinary Research, 57: 269-275.*

**Kidder H.E., Casida L.E., Grummer R.H.** (1955). Some effects of estrogen injections on the estrual cycle of gilts. *Journal of Animal Science, 14: 470-474.*

**Larsen J.C., Hunt J., Perrin I., Ruckenbauer P.** (2004). Workshop on trichothecenes with a focus on DON: summary report. *Toxicology Letters, 153: 1-22.*

**Malekinejad H., Maas-Bakker R.F., Fink-Gremmels J.** (2005). Bioactivation of zearalenone by porcine hepatic biotransformation. *Veterinary Research, 36: 799-810.*

**Malekinejad H., Schoevers E.J., Daemen I.J.J.M., Zijstra C., Colenbrander B., Fink-Gremmels J., Roelen B.A.J.** (2007). Exposure of oocytes to the *Fusarium* toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. *Biology of Reproduction, 77: 840-847.*

**Marasas W.F.O., Kellerman T.S., Gelderblom W.C., Coetzer J.A., Thiel P.G., van der Lugt J.J.** (1988). Leukoencephalomalacia in a horse induced by fumonisin $B_1$ isolated from *Fusarium moniliforme. Onderstepoort Journal of Veterinary Research, 55: 197-203.*

**Marasas W.F.O., Riley R.T., Hendricks K.A., Stevens V.L., Sadler T.W., Gelineau-van Waes J., Missmer S.A., Cabrera J., Torres O., Gelderblom W.C.A., Allegood J., Martìnez C., Maddox J., Miller J.D., Starr L., Sullards M., Roman A.V., Voss K.A., Wang E., Merrill A.H.** (2004). Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among populations consuming fumonisin- contaminated maize. *Journal of Nutrition, 134: 711-716.*

**Medvedova M., Kolesarova A., Capcarova M., Labuda R., Sirotkin A.V., Kovacik J., Bulla J.** (2011). The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells *in vitro. Journal of Environmental Science and Health - Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 46: 213-219.*

**Merrill A.H. Jr., Sullards M.C., Wang E., Voss K.A., Riley R.T.** (2001). Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives, 109: 283-289.*

**Minervini F., Dell'Aquila M.E.** (2008). Zearalenone and Reproductive Function in Farm Animals. *International Journal of Molecular Sciences, 9: 2570-2584.*

**Minervini F., Dell'Aquila M.E., Maritato F., Minoia P., Visconti A.** (2001). Toxic effects of the mycotoxin zearalenone and its derivatives on *in vitro* maturation of bovine oocytes and 17β-estradiol levels in mural granulosa cell cultures. *Toxicology in Vitro, 15: 489-495.*

**Minervini F., Giannoccaro A., Fornelli F., Dell'Aquila M.E., Minoia P., Visconti A.** (2006). Influence of mycotoxin zearalenone and its derivatives (alpha and beta zearalenol) on apoptosis and proliferation of cultured granulosa cells from equine ovaries. *Reproductive Biology and Endocrinology, 4: 62.*

**Minervini F., Lacalandra G.M., Filannino A., Garbetta A., Nicassio M., Dell'Aquila M.E., Visconti A.** (2010). Toxic effects induced by mycotoxin fumonisin $B_1$ on equine spermatozoa: assessment of viability, sperm chromatin structure stability, ROS production and motility. *Toxicology In Vitro, 24: 2072-2078.*

**Mirocha C.J., Pathre S.V., Christensen C.M.** (1977). Zearalenone. In: *Rodricks J.V., Hesseltine C.W., Mehlman M.A. (Eds.), Mycotoxins in Human and Animal Health, Pathotox Publisher Inc.: Park Forest South, IL, USA, pp. 346-364.*

**Morgavi D.P., Riley R.T.** (2007). A historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with *Fusarium* toxins. *Animal Feed Science and Technology, 137: 201-212.*

**Morooka N., Uratsuji N., Yoshizawa T., Yamamoto H.** (1972). Studies on the toxic effects of barley infected with *Fusarium* spp. *Journal of The Food Hygienic Society of Japan, 13: 368-375.*

**Mostrom M.S., Merl F., Raisbeck M.F.** (2012). Trichothecenes. In: *Gupta R.C. (Ed.), Veterinary Toxicology: Basic and Clinical Principles (second edition), Elsevier Inc., pp. 1239-1265.*

**Nagy C.M., Fejer S.N., Berek L., Molnar J., Viskolcz B.** (2005). Hydrogen bondings in deoxynivalenol (DON) conformations – a density functional study. *Journal of Molecular Structure, 726: 55-59.*

**Ndossi D.G., Frizzell C., Tremoen N.H., Fæste C.K., Verhaegen S., Dahl E., Eriksen G.S., Sørlie M., Connolly L., Ropstad E.** (2012). An *in vitro* investigation of endocrine disrupting effects of trichothecenes deoxynivalenol (DON), T-2 and HT-2 toxins. *Toxicology Letters, 214: 268-278.*

**Newman K.E., Raymond S.L.** (2005). Effects of mycotoxins in horses. In: *Diaz D.E. (Ed.), The Mycotoxin Blue Book, Nottingham University Press, Nottingham, UK, pp. 57-76.*

**Osweiler G.D., Ross P.F., Wilson T.M., Nelson P.E., Witte S.T., Carson T.L., Rice L.G., Nelson H.A.** (1992). Characterization of an epizootic of pulmonary edema in swine associated with fumonisin in corn screenings. *Journal of Veterinary Diagnostic Investigation, 4: 53-59.*

**Otokawa M.** (1983). Immunological disorders. In: *Ueno Y. (Ed.), Trichothecenes – Chemical, Biological, and Toxicological Aspects, vol. 4. Elsevier, New York, USA, pp. 163-170.*

**Pestka J.J.** (2007). Deoxynivalenol: toxicity, mechanisms and health risks. *Animal Feed Science and Technology, 137: 283-298.*

**Pestka J.J., Smolinski A.T.** (2005). Deoxynivalenol: toxicology and potential effects on humans. *Journal of Toxicology and Environmental Health. Part B, Critical Reviews, 8: 39-69.*

**Placinta C.M., D'Mello C.P.F., MacDonald A.M.C.** (1999). A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Animal Feed Science and Technology, 78: 21-37.*

**Qian Y., Shi W.Q., Ding J.T., Sha J.H., Fan B.Q.** (2003). Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilized *in vitro*. *The Journal of Reproduction and Development, 49: 167-174.*

**Ranzenigo G., Caloni F., Cremonesi F., Aad P.Y., Spicer L.J.** (2008). Effects of *Fusarium* mycotoxins on steroid production by porcine granulosa cells. *Animal Reproduction Science, 107: 115-130.*

**Richard J.L.** (2007). Some major mycotoxins and their mycotoxicoses – An overview. *International Journal of Food Microbiology, 119: 3-10.*

**Rocha O., Ansari K., Doohan F.M.** (2005). Effects of trichothecene mycotoxins on eukaryotic cells: a review. *Food Additives and Contaminants, 22: 369-378.*

**Rodrigues I., Naehrer K.** (2012). A Three-Year Survey on the Worldwide Occurrence of Mycotoxins in Feedstuffs and Feed. *Toxins, 4: 663-675.*

**Rotter B.A., Prelusky D.B., Pestka J.J.** (1996). Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health, 48: 1-34.*

**Schoevers E.J., Fink-Gremmels J., Colenbrander B., Roelen B.A.** (2010). Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. *Theriogenology, 74: 968-978.*

**Smith G.W.** (2012). Fumonisins. In: *Gupta R.C. (Ed.), Veterinary Toxicology: Basic and Clinical Principles (second edition), Elsevier Inc., pp. 1205-1219.*

**Smith G.W., Constable P.D., Foreman J.H., Eppley R.M., Waggoner A.L., Tumbleson M.E., Haschek W.M.** (2002). Cardiovascular changes associated with intravenous administration of fumonisin $B_1$ in horses. *American Journal of Veterinary Research, 63: 538-545.*

**Sobrova P., Adam V., Vasatkova A., Beklova M., Zeman L., Kizek R.** (2010). Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology, 3: 94-99.*

**Sweeney M.J., Dobson A.D.W.** (1998). Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *International Journal of Food Microbiology, 43: 141-158.*

**Sydenham E.W., Shephard G.S., Thiel P.G., Marasas W.F.O., Stockestrom S.** (1991). Fumonisin contamination of commercial corn-based human foodstuffs. *Journal of Agricultural and Food Chemistry, 39: 2014-2018.*

**Takagi M., Mukai S., Kuriyagawa T., Takagaki K., Uno S., Kokushi E., Otoi T., Budiyanto A., Shirasuna K., Miyamoto A., Kawamura O., Okamoto K., Deguchi E.** (2008). Detection of zearalenone and its metabolites in naturally contaminated follicular fluids by using LC/MS/MS and *in vitro* effects of zearalenone on oocyte maturation in cattle. *Reproductive Toxicology, 26: 164-169.*

**Tiemann U., Dänicke S.** (2007). *In vivo* and *in vitro* effects of the mycotoxins zearalenone and deoxynivalenol on different non-reproductive and reproductive organs in female pigs: a review. *Food Additives and Contaminants, 24: 306-314.*

**Tiemann U., Tomek W., Schneider F., Vanselow J.** (2003). Effects of the mycotoxins α- and β-zearalenol on regulation of progesterone synthesis in cultured granulosa cells from porcine ovaries. *Reproductive Toxicology, 17: 673-681.*

**Towers N.R., Sprosen J.M.** (1993). Zearalenone-induced infertility in sheep and cattle in New Zealand. *New Zealand Veterinary Journal, 41: 223-224.*

**Ueno Y.** (1983). Trichothecenes: Chemical, biological, and toxicological aspects. In: *Ueno Y. (Ed.), Trichothecenes, Elsevier Press, Amsterdam, pp. 135-146.*

**Venturini M.C., Quiroga M.A., Risso M.A., Di Lorenzo C., Omata Y., Venturini L., Godoy H.** (1996). Mycotoxin T-2 and Aflatoxin $B_1$ as immunosuppressors in mice chronically infected with Toxoplasma gondii. *Journal of Comparative Pathology, 115: 229-237.*

**Vesonder R.F., Ciegler A., Jensen A.H.** (1973). Isolation of the emetic principle from *Fusarium*-infected corn. *Applied Microbiology, 26: 1008-1010.*

**Voss K.A., Chamberlain W.J., Bacon C.W., Norred W.P.** (1993). A preliminary investigation on renal and hepatic toxicity in rats fed purified fumonisin $B_1$. *Natural Toxins, 1: 222-228.*

**Voss K.A., Smith G.W., Haschek W.M.** (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. *Animal Feed Science and Technology, 137: 299-325.*

**Wang E., Norred W.P., Bacon C.W., Riley R.T., Merrill A.H. Jr.** (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *The Journal of Biological Chemistry, 266: 14486-14490.*

**Wang E., Ross P.F., Wilson T.M., Riley R.T., Merrill A.H.** (1992). Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. *Journal of Nutrition, 122: 1706-1716.*

**Weaver G.A., Kurtz H.J., Behrens J.C., Robison T.S., Seguin B.E., Bates F.Y., Mirocha C.J.** (1986). Effect of zearalenone on the fertility of virgin dairy heifers. *American Journal of Veterinary Research, 47: 1395-1397.*

**Yazar S., Omurtag G.Z.** (2008). Fumonisins, Trichothecenes and Zearalenone in Cereals. *International Journal of Molecular Sciences, 9: 2062-2090.*

**Yoshizawa T., Morooka N.** (1973). Deoxynivalenol and its monoacetate: New mycotoxins from *Fusarium roseum* and moldy barley. *Agricultural and Biological Chemistry, 37: 2933-2934.*

**Young L.G., King G.J.** (1986). Low concentrations of zearalenone in diets of mature gilts. *Journal of Animal Science, 63: 1191-1196.*

**Young L.G., Ping H., King G.J.** (1990). Effects of feeding zearalenone to sows on rebreeding and pregnancy. *Journal of Animal Science, 68: 15-20.*

**Zhu L., Yuan H., Guo C., Lu Y., Deng S., Yang Y., Wei Q., Wen L., He Z.** (2012). Zearalenone induces apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9-dependent mitochondrial signaling pathway. *Journal of Cellular Physiology, 227: 1814-1820.*

**Zinedine A., Soriano J.M., Moltó J.C., Mañes J.** (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food and Chemical Toxicology, 45: 1-18.*

# CHAPTER 2

## Objectives

# 2. Objectives

There is now overwhelming evidence of global contamination of cereals and animal feeds with fumonisins, trichothecenes and zearalenone (ZEA), the major mycotoxins of *Fusarium* fungi. Of these mycotoxins, ZEA is unequivocally implicated in reproductive disorders of swine and other domestic animals. Experiments *in vivo* and *in vitro* indicate that ZEA and its metabolites exert estrogenic-like actions due to their binding affinity to estrogen receptors and thus influence the reproductive system and are grouped with the compounds known collectively as endocrine disrupters.

Recently, the potential of trichothecenes to act as endocrine disruptors has been investigated. *In vitro* studies have shown that DON and T-2 toxin have potent dose-dependent effects on porcine granulosa cell steroid production and proliferation and that DON inhibits porcine oocyte maturation. Moreover, animal studies have shown abnormal reproductive effects of T-2 toxin.

So far very little information is available on the potential reproductive toxicity of fumonisins. Previous study demonstrated that fumonisin $B_1$ ($FB_1$), the most significant of the fumonisins in terms of toxicity and occurrence, delays puberty and impairs semen quality and spermatogenesis in pigs and rabbits, with potential to impair fertility. Moreover, $FB_1$ was found to affect *in vitro* some functional parameters of equine spermatozoa, such as sperm chromatin stability and motility. Until now, no study has evaluated direct effects of $FB_1$ on the ovary. Therefore, the aim of the present study was to evaluate *in vitro* if $FB_1$ can impair swine reproductive function *via* affecting granulosa cell proliferation (chapter 3), steroidogenesis and gene expression (chapter 4). Because co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is a recurring feature of several surveys, the interaction between $FB_1$ and DON or α-zearalenol (α-ZOL), ZEA major active metabolite, was also investigated.

# CHAPTER 3

# Effects of fumonisin $B_1$ and deoxynivalenol or zearalenone on porcine granulosa cell proliferation

# 3. Effects of fumonisin $B_1$ alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation

## 3.1. Abstract

Fumonisins are mycotoxins mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum* occurring on a worldwide basis in corn. Fumonisin $B_1$ ($FB_1$) is the most abundant and toxic and causes a variety of species-specific toxic effects in domestic animals *e.g.* equine leukoencephalomalacia and porcine pulmonary edema. So far, very little information is available on the potential reproductive toxicity of $FB_1$. Thus, the goal of this study was to determine if $FB_1$, alone or combined with deoxynivalenol (DON) or α-zearalenol (α-ZOL), zearalenone major active metabolite, can impair pig reproductive functions *via* affecting granulosa cell proliferation. Porcine granulosa cells from small follicles (1-5 mm) were cultured for 2 days in 5% fetal bovine serum and 5% porcine serum-containing medium followed by 2 days in serum-free medium with or without added treatments. The results revealed that $FB_1$ had inhibitory effects on granulosa cell proliferation at doses ≥ 10 μM. DON (3.4 μM) strongly inhibited granulosa cell growth and no significant difference was detected in combination with $FB_1$. α-ZOL (9.4 μM) showed a stimulatory effect on granulosa cell numbers even in combination with $FB_1$. The present study indicates that $FB_1$ has direct effects on proliferation of porcine granulosa cell and thus may be able to alter growth of the granulosa layer within ovarian follicles. The interaction between $FB_1$ and DON or ZEA was also evaluated. No additive or synergistic effects were observed after combined exposure to DON and $FB_1$ or α-ZOL and $FB_1$.

## 3.2. Introduction

*Fusarium* mycotoxins, which are commonly found on cereals grown in the northern hemisphere (Creppy, 2002), include trichothecenes, such as deoxynivalenol (DON) and T-2 toxin, zearalenone and fumonisins, and have been implicated in poor reproductive performance in pigs (Alm et al., 2002, 2006; Malekinejad et al., 2007; Gbore and Egbunikem, 2008; Ranzenigo et al., 2008; Caloni et al., 2009; Gbore, 2009a, 2009b; Schoevers et al., 2010).
Fumonisins are mycotoxins mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum* that have been shown to occur worldwide at significant

levels in corn and corn by-products used in animal feeds (Sydenham et al., 1991). Although several natural fumonisins are known, fumonisin $B_1$ ($FB_1$) has been reported to be the most abundant and toxic followed by fumonisin $B_2$ ($FB_2$) and fumonisin $B_3$ ($FB_3$) (Murphy et al., 1993; Norred, 1993).

$FB_1$ has a species-specific toxicity in domestic and laboratory animals as it induces porcine pulmonary edema (PPE) in swine (Harrison et al., 1990; Osweiler et al., 1992; Colvin et al., 1993), equine leukoencephalomalacia (ELEM) in horses (Marasas et al., 1988; Kellerman et al., 1990), hepatocarcinoma in rats (Gelderblom et al., 1991) and mice (Howard et al., 2001a), nephrocarcinoma in rats (Howard et al., 2001b), and nephropathy in rats (Voss et al., 1993) and rabbits (Gumbrecht et al., 1995). Moreover, experimentally $FB_1$ showed a liver toxicity in many species including pigs (Haschek et al., 1992; Casteel et al., 1993), horses (Ross et al., 1993), cattle (Edrington et al., 1995), rats (Voss et al., 1989) and primates (Jaskiewicz et al., 1987). $FB_1$ is also toxic to the cardiovascular system in pigs and horses (Voss et al., 2007). In pigs, $FB_1$ decreases cardiac contractility, mean systemic arterial pressure, heart rate and cardiac output, and increases mean pulmonary artery pressure and pulmonary artery wedge pressure (Constable et al., 2000, 2003). Therefore, $FB_1$-induced PPE appears to result from acute left-sided heart failure (Smith et al., 1999; Voss et al., 2007). Clinical signs associated with the development of PPE consistently begin 3-6 days after initiation of exposure to high concentration of $FB_1$ and include dyspnea, increased respiratory rate, weakness, cyanosis and sudden death (Smith, 2012). In pigs, hepatic toxicity occurs prior to the development of PPE and alterations are time and dose dependent (Motelin et al., 1994). Effects of $FB_1$ on both specific and non-specific immunity of pigs have also been reported (Casteel et al., 1993; Harvey et al., 1995, 1996).

Studies on the reproductive and developmental toxicity of $FB_1$ are rare. $FB_1$ was reported to negatively affect the development of rat (Flynn et al., 1996) and chicken (Javed et al., 1993; Zacharias et al., 1996) embryo and to be detrimental to fetal survivability of Syrian hamsters (Floss et al., 1994). Recently, dietary exposure to $FB_1$ was found to delay puberty and to reduce sperm production and impair semen quality in pigs (Gbore and Egbunikem, 2008; Gbore, 2009a; Gbore, 2009b) and rabbits (Ewuola and Egbunike, 2010). Moreover, *in vitro,* $FB_1$ affects some functional parameters of equine spermatozoa, such as sperm chromatin stability and motility, with potential to determine subfertility in stallion (Minervini et al., 2010). Because reduced reproductive performance in livestock can have a significant economic impact, further investigations on reproductive effects of $FB_1$ are needed. Previous *in vitro* studies demonstrated that other *Fusarium* mycotoxins such as zearalenone, DON and T-2 toxin may alter steroidogenesis and be able to alter growth of the granulosa cell layer within ovarian follicles in swine (Ranzenigo et al., 2008; Caloni et al., 2009; Medvedova

et al., 2011; Zhu et al., 2012). Until now, no study has evaluated direct effects of $FB_1$ on the ovary. Therefore, the aim of the present study was to assess if $FB_1$ alone or in combination with other *Fusarium* mycotoxins such as DON or zearalenone can impair pig reproductive functions *via* affecting granulosa cell proliferation.

## 3.3. Materials and methods

### *3.3.1. Reagents and Hormones*

Dulbecco's Modified Eagle Medium (DMEM), Ham's F-12, fetal calf serum (FCS), porcine serum (PS), gentamicin, L-glutamine, sodium bicarbonate, trypan blue, collagenase, DNase, $FB_1$, α-zearalenol (α-ZOL) and DON were all purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Recombinant human insulin-like growth factor-I (IGF-I) and recombinant human fibroblast growth factor-9 (FGF9) were obtained from R&D Systems (Minneapolis, MN). Ovine follicle-stimulating hormone (FSH; 175 x NIH-FSH-S1 U/mg) was obtained from Dr. A. F. Parlow, National Hormone & Pituitary Program (Torrance, CA) and testosterone from Steraloids (Wilton, NH).

### *3.3.2. Cell Culture*

Ovaries were obtained from non-pregnant gilts slaughtered at a nearby commercial abattoir and were treated as previously described (Spicer and Hammond, 1987, 1989; Ranzenigo et al., 2008). The ovaries were washed three times in 0.9% saline, immersed in 70% ethanol for 30 s, washed again three times with 0.9% saline and transported to the laboratory in 0.9% saline with 1% streptomycin/penicillin on ice. Follicle diameters (longitudinal and transverse planes) were measured with a caliper (Figure 3.1) and the mean diameter was calculated. Follicular fluid was aspirated aseptically from small follicles (1-5 mm) with a 25 gauge needle (Figure 3.2) and granulosa cells were recovered by centrifugation at 200 x *g* for 8 min. Granulosa cells were washed twice with 7 mL of serum-free medium; at each wash, cells were separated from medium via centrifugation (200 x *g* at 4° C for 7-8 min). After the last centrifugation the supernatant was aspirated and replaced with 2 mL of enzyme-containing medium (0.5 mg/mL of DNase and 1.25 mg/mL of collagenase) to prevent clumping of cells as previously described (Spicer et al., 2002). Numbers of viable cells were determined using the trypan blue exclusion method (Langhout et al., 1991; Spicer et al., 1993; Tiemann et al., 2003).

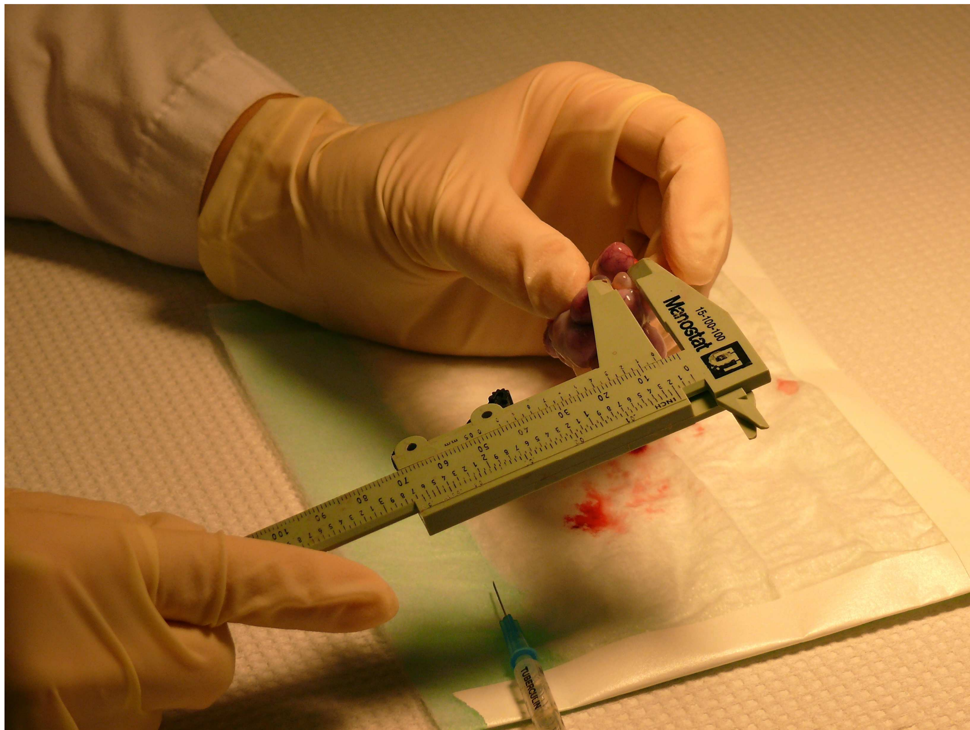



**Figure 3.1.** Measurement of follicle diameters (longitudinal and transverse planes) with a caliper. Only follicles with a mean diameter ≤ 5 mm were used to collect granulosa cells.

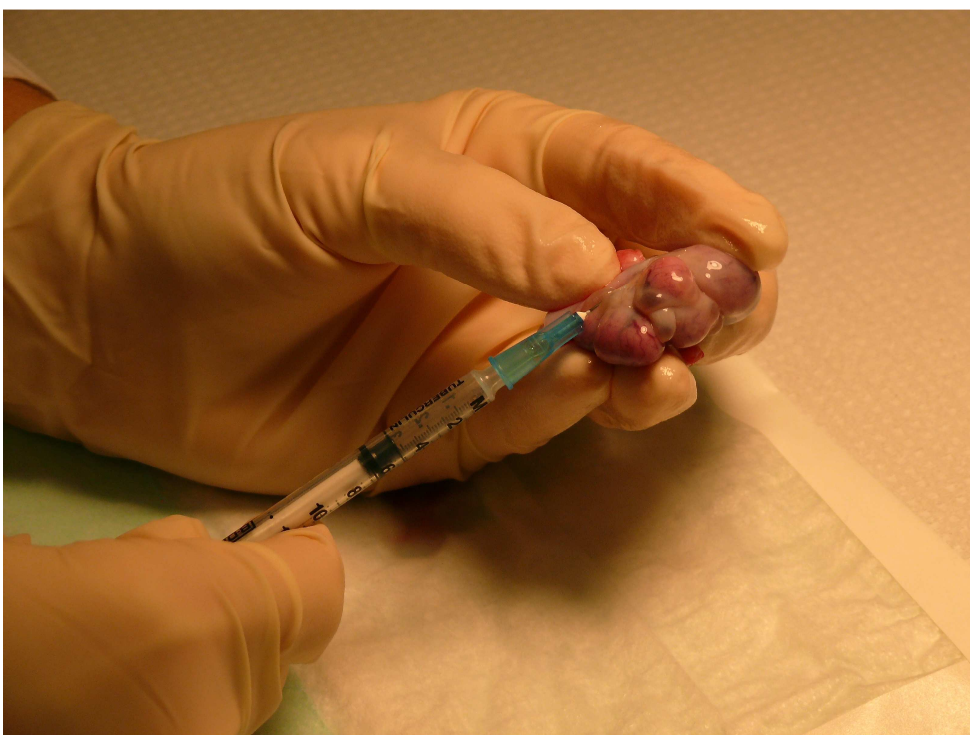


**Figure 3.2.** Collection of granulosa cells from small porcine follicles (1-5 mm) via needle aspiration.

Viability of porcine granulosa cells from small follicles averaged 32 ± 2.1% at the time of plating. Between 2.9 and 3.7 x $10^5$ viable cells were plated on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ) in 1 mL of medium composed of a mixture of 1:1 DMEM and Ham's F-12 containing glutamine (2 mM), gentamicin (0.12 mM) and sodium bicarbonate (38.5 mM). Cultures were kept at 38.5° C in a humidified 95% air-5% $CO_2$ environment, and medium was changed every 24 h. To obtain an optimal attachment, cells were maintained in the presence of 5% FCS and 5% PS for the first 48 h of culture. After this time, granulosa cells were washed twice with 0.5 mL of serum-free medium and incubations continued in serum-free medium for 2 days containing 500 ng/mL of testosterone (as an estradiol precursor; Spicer and Chamberlain, 1998; Ranzenigo et al., 2008) with or without added treatments as described below.

### *3.3.3. Determination of granulosa cell numbers*

Numbers of granulosa cells were determined at the end of experiments using a Coulter counter (Z2 Coulter particle count and size analyzer; Beckman Coulter, Hialeah, FL) (Figure 3.3).

**Figure 3.3.** Determination of granulosa cell numbers using a Z2 Coulter® Particle Count and Size Analyzer.

Briefly, wells were washed twice with 0.9% saline solution (500 μL); after the second wash, cells were exposed to 0.5 mL of trypsin (0.25% wt/vol=2.5 mg/mL) for 20 min at room temperature. After this time, cells were scraped from each well with a teflon policeman (Figure 3.4), diluted 1:10 in 0.9% saline solution and enumerated as previously described (Spicer and Hammond, 1987; Langhout et al., 1991; Ranzenigo et al., 2008). Cell aggregates were minimized by pipetting cell suspensions back and forth through a 500 μL pipette tip three to five times.

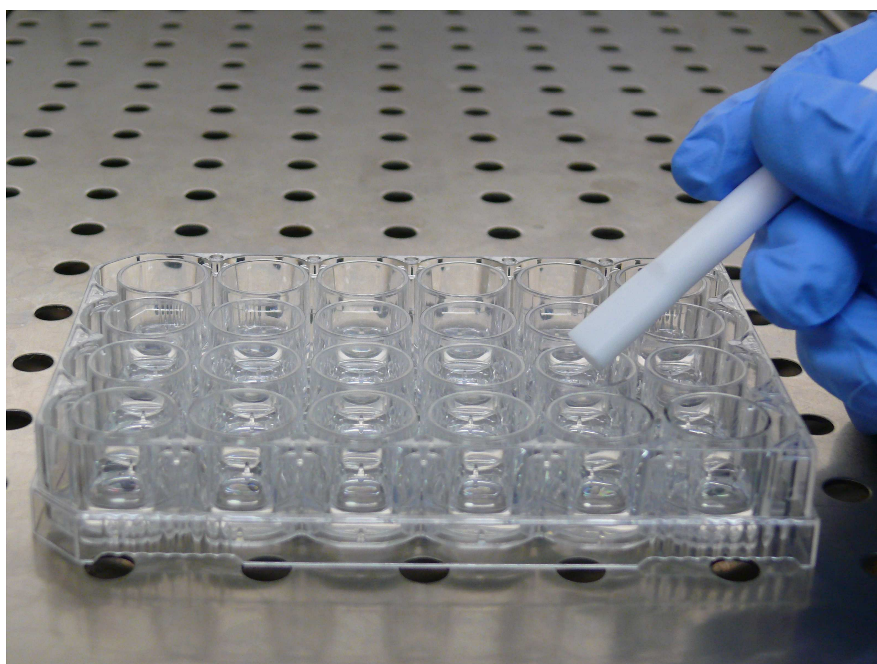


**Figure 3.4.** Cell scraping with a teflon policeman. After 20 min incubation with trypsin at room temperature, cells were scraped from each well with a teflon policeman, diluted 1:10 in 0.9% saline solution and enumerated.

### *3.3.4. Experimental design*

Experiment 1 was designed to identify the dose-response of $FB_1$ on granulosa cell proliferation in the presence of FSH plus IGF-I or FGF9 or both. Cells were cultured for 48 h in 5% FCS and 5% PS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing FSH (30 ng/mL), IGF-I (0 or 30 ng/mL) and FGF9 (0 or 30 ng/mL) with or without the various doses of $FB_1$ (i.e., 0.01, 0.4 and 14 μM). After 48 h of treatment, granulosa cells were counted.
Experiment 2 was designed to evaluate the effects of DON and α-ZOL with and without $FB_1$ on FSH plus IGF-I-induced granulosa cell proliferation. Cells were cultured for 48 h in 5% FCS and 5% PS, washed twice with serum-free medium

as described earlier, and cells treated for 48 h in serum-free medium containing 30 ng/mL of IGF-I and 30 ng/mL of FSH with or without $FB_1$ (10 μM), DON (3.4 μM) and α-ZOL (9.4 μM). After 48 h of treatment, granulosa cells were counted.
Experiment 3 was designed to determine the effect of $FB_1$ on serum-stimulated granulosa cell proliferation. Cells were cultured for 4 days in 5% FCS and 5% PS. During the last 2 days of culture, cells were treated as follows: control (no additions) or $FB_1$ (10 μM). At the end of treatment, granulosa cells were counted.

### *3.3.5. Statistical analysis*

Experimental data are presented as the least squares means ± SEM of measurements from replicated culture wells. Each experiment was performed three times with different pools of granulosa cells collected from 2 to 6 ovaries for each pool and each treatment replicated three times within each experiment. Treatment effects and interactions were assessed using the GLM procedure of the Statistical Analysis System (SAS, 1999). Main effects were treatment, experiment, and their interaction when data from more than one experiment were analyzed. Mean differences in cell growth between treatments were determined using the Fisher's protected least significant difference (LSD) procedure (Ott, 1977).

## 3.4. Results

### *3.4.1. Experiment 1: effects of $FB_1$ on granulosa cell proliferation in the presence of FSH plus IGF-I or FGF9 or both.*

Experiment 1 was conducted to identify the dose-response of $FB_1$ on granulosa cell proliferation in the presence of FSH plus IGF-I or FGF9 or both. All doses of $FB_1$ (i.e., 0.01, 0.4 and 14 μM) had no significant effect ($P > 0.10$) on granulosa cell numbers in the presence of FSH (control cultures) (Figure 3.5). At 14 μM $FB_1$ decreased ($P < 0.05$) cell numbers by 20% and 32% in IGF-I- and FGF9-treated cultures, respectively, whereas cell numbers were not significantly affected ($P > 0.10$) by 0.01 μM and 0.4 μM of $FB_1$ (Figure 3.5). In IGF-I plus FGF9-treated cultures, at 0.4 μM and 14 μM $FB_1$ decreased ($P < 0.05$) cell numbers by 18% and 45%, respectively (Figure 3.5).

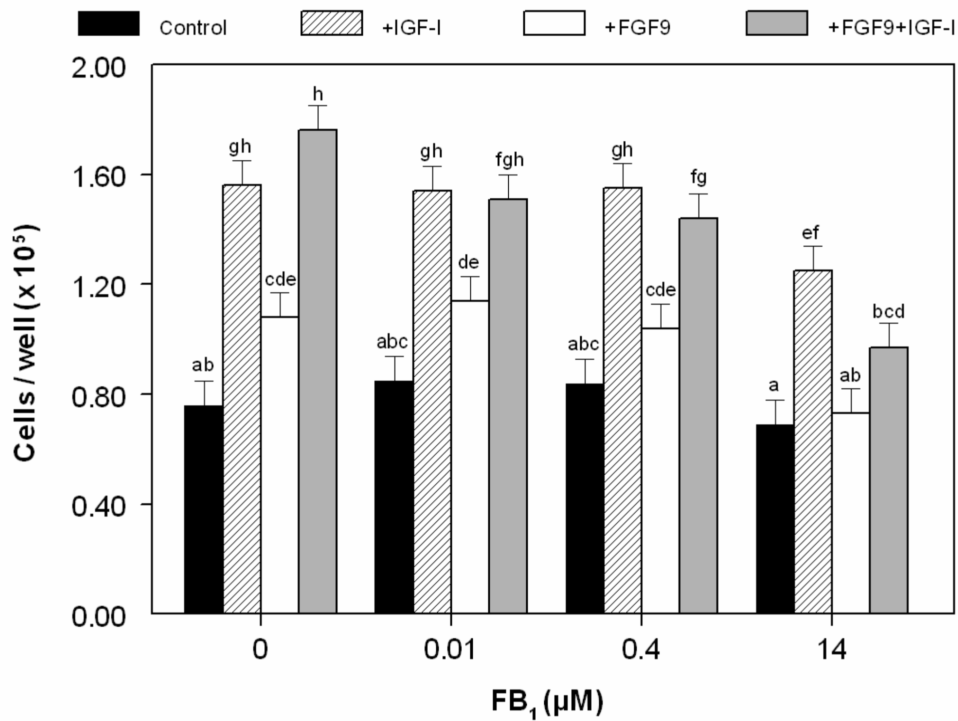



**Figure 3.5.** Effect of $FB_1$ on numbers of granulosa cells from porcine follicles (Experiment 1). Granulosa cells were cultured for 4 days as described in Materials and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL), IGF-I (0 or 30 ng/mL) and FGF9 (0 or 30 ng/mL) with or without the various doses of $FB_1$. Values are means from three separate experiments ($n$ = 9). Means without a common letter (a-h) differ ($P < 0.05$).

### *3.4.2. Experiment 2: effects of DON and a-ZOL with and without $FB_1$ on FSH plus IGF-I-induced granulosa cell proliferation*

Experiment 2 was conducted to determine the interaction between $FB_1$ and DON or α-ZOL on FSH plus IGF-I-induced granulosa cell proliferation. DON (3.4 μM) strongly inhibited (by 80%; $P < 0.0001$) granulosa cell proliferation and no significant difference was detected in combination with $FB_1$ (10 μM) (Figure 3.6). Alone, $FB_1$ showed an inhibitory effect ($P < 0.05$) on granulosa cell proliferation; cell numbers were decreased by 21% (Figure 3.6). α-ZOL (9.4 μM) showed a stimulatory effect ($P < 0.01$) on granulosa cell numbers, even when treated in combination with $FB_1$ (Figure 3.6).

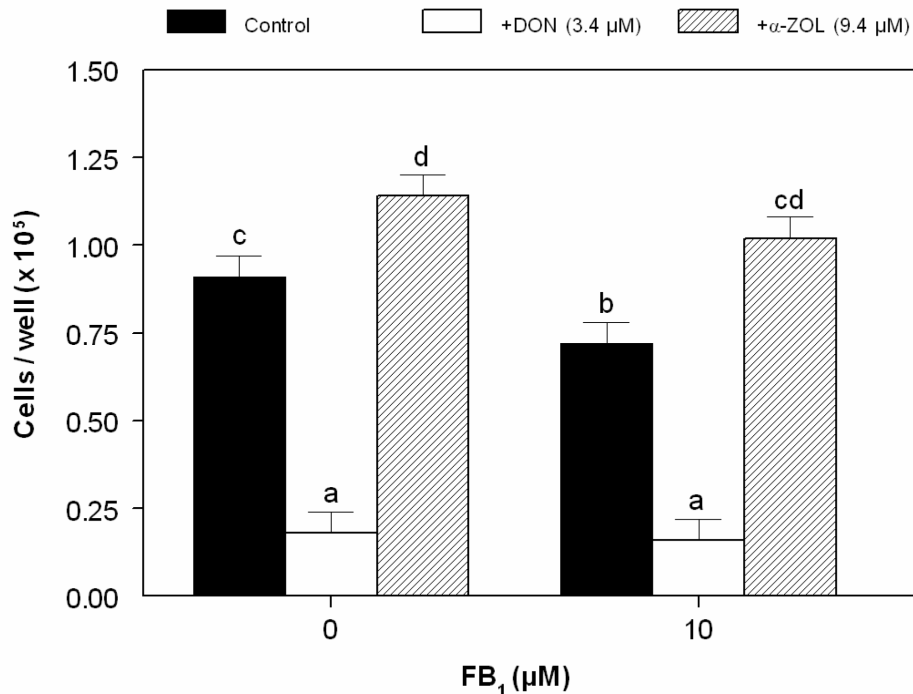



**Figure 3.6.** Interaction between $FB_1$ and DON or α-ZOL on FSH plus IGF-I-induced proliferation of porcine granulosa cells (Experiment 2). Granulosa cells were cultured for 4 days as described in Materials and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing 30 ng/mL of IGF-I and 30 ng/mL of FSH with or without $FB_1$ (10 µM), DON (3.4 µM) and α-ZOL (9.4 µM). Values are means from three separate experiments (*n* = 9). Means without a common letter (a-d) differ ($P < 0.05$).

### *3.4.3. Experiment 3: effect of $FB_1$ on serum-induced granulosa cell proliferation*

Experiment 3 was conducted to evaluate the effect of $FB_1$ on serum-stimulated granulosa cell proliferation. Alone, $FB_1$ (10 µM) decreased ($P < 0.005$) granulosa cell proliferation induced by 5% FCS plus 5% PS (Figure 3.7). Cell numbers were decreased ($P < 0.005$) by 29% after 1 day and by 38% after 2 days of $FB_1$ treatment (Figure 3.7).

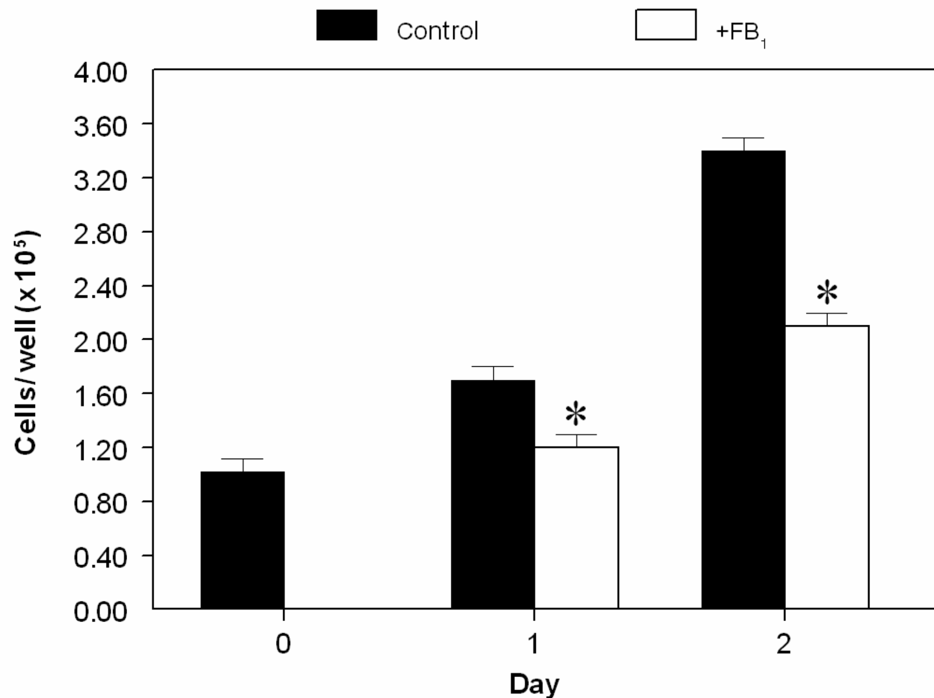



**Figure 3.7.** Effect of $FB_1$ on serum-induced proliferation of porcine granulosa cells (Experiment 3). Granulosa cells were cultured for 4 days as described in Materials and Methods. During the last 2 days of culture, cells were treated with 5% FCS plus 5% PS medium with no additions or 10 μM $FB_1$; within day of treatment, single asterisk (*) indicates mean differs ($P < 0.005$) from Control value. Values are means from three separate experiments ($n = 9$).

## 3.5. Discussion

Despite the overwhelming evidence of global contamination of corn and animal feed with fumonisins (Placinta et al., 1999; Binder et al., 2007), data on reproductive toxicity induced by $FB_1$ are very limited. To our knowledge, effects of $FB_1$ on granulosa cells have not been previously investigated in any species. Considering that pigs are high consumers of corn and are thus particularly exposed to fumonisins (Colvin and Harrison, 1992), in the present study pig granulosa cells were used to determine the effect of $FB_1$ on pig reproductive functions.

The results revealed that: (1) $FB_1$ had inhibitory effects on granulosa cell proliferation; (2) cell growth was more sensitive to inhibition by $FB_1$ in the presence of FSH plus IGF-I and FGF9; (3) DON strongly inhibited granulosa cell proliferation and no significant difference was detected in combination with

$FB_1$; (4) α-ZOL showed a stimulatory effect on granulosa cell numbers even in combination with $FB_1$; and (5) $FB_1$ alone inhibited cell growth induced by serum.

An inhibitory effect of $FB_1$ was observed on granulosa cell proliferation as measured by decreased cell numbers at doses $\geq$ 10 μM. Comparable results were previously obtained with porcine epithelial cell lines of renal (LLC-PK1) and intestinal (IPEC-1) origins. Yoo et al. (1992) showed an inhibition of LLC-PK1 proliferation with $FB_1$ concentrations ranging from 10 to 35 μM. In a subsequent study, Bouhet et al. (2004) demonstrated that concentrations $\geq$ 10 μM $FB_1$ inhibit the proliferation of IPEC-1 and LLC-PK1 by blocking them in the G0/G1 phase of the cell cycle. Cells blocked in the phase G0/G1 have a finite proliferative potential and could not follow a normal cell cycle. A blockade of the cell cycle by $FB_1$ has been also reported in primary cells such as porcine lymphocytes (Marin et al., 2007). This arrest in cell cycle was observed with a very low dose of $FB_1$ (1 μM) and was associated with a dose-dependent anti-proliferative effect observed at concentrations $\geq$ 10 μM (Marin et al., 2007).

The mechanism by which $FB_1$ inhibits porcine granulosa cell proliferation will require elucidation but based on the previous studies (Yoo et al., 1992; Yoo et al., 1996; Riley et al., 1999) conducted with LLC-PK1, a cell line, which is considered to be a good model for studying $FB_1$ *in vitro* effects, there is a close correlation between fumonisin-induced disruption of sphingolipid metabolism and inhibition of cell growth. $FB_1$ bears a remarkable structural resemblance to the long-chain (sphingoid) base backbones of sphingolipids and thus impairs sphingolipid biosynthesis by specifically inhibiting ceramide synthase (Wang et al., 1991; Merrill et al., 2001; Voss et al., 2007). The inhibition of ceramide synthase leads to the accumulation of sphingoid bases (sphinganine and, to a lesser degree, sphingosine) and to the depletion of complex sphingolipids (Wang et al., 1991; Merrill et al., 2001; Voss et al., 2007). Sphingoid bases are known to mediate several key biological processes such as cell proliferation or DNA replication (Spiegel and Merrill, 1996). Using myriocin, a potent and selective inhibitor of the enzyme serine palmitoyltransferase, which catalyzes the first step in the sphingolipid biosynthetic pathway, the antiproliferative effect of $FB_1$ was found to be mainly due to the accumulation of free sphinganine in LLC-PK1 cells (Riley et al., 1999). The accumulation of sphingoid bases is considered the primary cause of the toxicity of $FB_1$; however the full effects of $FB_1$ probably involve many biochemical events (Merrill et al., 2001).

The co-occurrence of *Fusarium* mycotoxins in commodities (Placinta et al., 1999) has become a significant issue with complex and, thus far, indeterminate implications for animal health and productivity. Therefore, we also investigated

the interaction between $FB_1$ and DON or α-ZOL. The effects of DON and α-ZOL on porcine granulosa cell function have been previously reported (Tiemann et al., 2003; Ranzenigo et al., 2008; Medvedova et al., 2011). In the present study, DON at 3.4 μM (1000 ng/mL) strongly inhibited FSH plus IGF-I-induced proliferation of granulosa cells. These results are in accordance with the study conducted by Ranzenigo et al. (2008), in which DON had a biphasic effect on cell growth with 10 and 100 ng/mL increasing cell numbers and 1000 ng/mL drastically inhibiting cell numbers. On the contrary, Medvedova et al. (2011) observed that DON at the dose of 1000 ng/mL increased the expression of markers of proliferation (cyclin B1 and PCNA) in porcine granulosa cells; however this occurred at shorter incubation times (24 h) than in the present study (48 h). DON was much more potent than $FB_1$ at inhibiting FSH plus IGF-I-induced cell growth. The inhibitory effect of DON on cell numbers was not affected by $FB_1$ as no significant difference was detected in combination with $FB_1$. In the present study, α-ZOL (9.4 μM) showed a stimulatory effect on granulosa cell numbers even in combination with $FB_1$ but these results differ from those obtained by other authors (Tiemann et al., 2003; Ranzenigo et al., 2008) in which α-ZOL showed no effect on cell numbers at 9.4 μM (Ranzenigo et al., 2008) and at 7.5-15 μM (Tiemann et al., 2003).

In conclusion, results of the present study indicate that $FB_1$ has direct and potent effects on granulosa cell proliferation and are the first to evaluate the effect of $FB_1$ on other *Fusarium* mycotoxins in granulosa cells. The consequences of these direct ovarian effects induced by *Fusarium* mycotoxins and their interactions could be considered in swine reproductive failures.

## 3.6. References

**Alm H., Brussow K.P., Torner H., Vanselow J., Tomek W., Danicke S., Tiemann U.** (2006). Influence of *Fusarium*-toxin contaminated feed on initial quality and meiotic competence of gilt oocytes. *Reproductive Toxicology, 22: 44-50.*

**Alm H., Greising T., Brüssow K.P., Torner H., Tiemann U.** (2002). The influence of the mycotoxins deoxynivalenol and zearalenol on *in vitro* maturation of pig oocytes and *in vitro* culture of pig zygotes. *Toxicology In Vitro, 16: 643-648.*

**Binder E.M., Tan L.M., Chin L.J., Handl J., Richard J.** (2007). Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal Feed Science and Technology, 137: 265-282.*

**Bouhet S., Hourcade E., Loiseau N., Fikry A., Martinez S., Roselli M., Galtier P., Mengheri E., Oswald I.P.** (2004). The mycotoxin fumonisin $B_1$ alters the proliferation and the barrier function of porcine intestinal epithelial cells. *Toxicological Sciences, 77: 165-171.*

**Caloni F., Ranzenigo G., Cremonesi F., Spicer L.J.** (2009). Effects of a trichothecene, T-2 toxin, on proliferation and steroid production by porcine granulosa cells. *Toxicon, 54: 337-344.*

**Casteel S.W., Turk J.R., Cowart R.P., Rottinghaus G.E.** (1993). Chronic toxicity of fumonisin in weanling pigs. *Journal of Veterinary Diagnostic Investigation, 5: 413-417.*

**Colvin B.M., Cooley A.J., Beaver R.W.** (1993). Fumonisin toxicosis in swine: clinical and pathologic findings. *Journal of Veterinary Diagnostic Investigation, 5: 232-241.*

**Colvin B.M., Harrison L.R.** (1992). Fumonisin-induced pulmonary edema and hydrothorax in swine. *Mycopathologia, 117: 79-82.*

**Constable P.D., Smith G.W., Rottinghaus G.M., Haschek W.M.** (2000). Ingestion of fumonisin $B_1$-containing culture material decreases cardiac contractility and mechanical efficiancy in swine. *Toxicology and Applied Pharmacology, 162: 151-160.*

**Constable P.D., Smith G.W., Rottinghaus G.E., Tumbleson M.E., Haschek W.M.** (2003). Fumonisin-induced blockade of ceramide synthase in sphingolipid biosynthetic pathway alters aortic input impedence spectrum of pigs. *American Journal of Physiology - Heart and Circulatory Physiology, 284: H2034-H2044.*

**Creppy E.E.** (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127: 19-28.*

**Edrington T.S., Kamps-Holtzapple C.A., Harvey R.B., Kubena L.F., Elissalde M.H., Rottinghaus G.E.** (1995). Acute hepatic and renal toxicity in lambs dosed with fumonisin-containing culture material. *Journal of Animal Science, 73: 508-515.*

**Ewuola E.O., Egbunike G.N.** (2010). Effects of dietary fumonisin $B_1$ on the onset of puberty, semen quality, fertility rates and testicular morphology in male rabbits. *Reproduction, 13: 439-445.*

**Floss J.L., Casteel S.W., Johnson G.C., Rottinghaus G.E., Krause G.F.** (1994). Development toxicity of fumonisin in Syrian hamsters. *Mycopathologia, 128: 33-38.*

**Flynn T.J., Pritchard D., Bradlaw J., Eppley R., Page S.** (1996). *In vitro* embryotoxicity of fumonisin $B_1$ evaluated with cultured postimplantation staged rat embryos. *In Vitro Toxicology, 9: 271-279.*

**Gbore F.A.** (2009a). Growth performance and puberty attainment in growing pigs fed dietary fumonisin $B_1$. *Journal of Animal Physiology and Animal Nutrition, 93: 761-767.*

**Gbore F.A.** (2009b). Reproductive organ weights and semen quality of pubertal boars fed dietary fumonisin $B_1$. *Animal, 3: 1133-1137.*

**Gbore F.A., Egbunike G.N.** (2008). Testicular and epididymal sperm reserves and sperm production of pubertal boars fed dietary fumonisin $B_1$. *Animal Reproduction Science, 105: 392-397.*

**Gelderblom W.C., Kriek N.P., Marasas W.F.O., Thiel P.G.** (1991). Toxicity and carcinogenicity of the Fusarium moniliforme metabolite, fumonisin $B_1$, in rats. *Carcinogenesis, 12: 1247-1251.*

**Gumprecht L.A., Marcucci A., Weigel R.M., Vesonder R.F., Riley R.T., Showker J.L., Beasley V.R., Haschek W.M.** (1995). Effects of intravenous fumonisin $B_1$ in rabbits: nephrotoxicity and sphingolipid alterations. *Natural Toxins, 3: 395-403.*

**Harrison L.R., Colvin B.M., Greene J.T., Newman L.E., Cole J.R. Jr.** (1990). Pulmonary edema and hydrothorax in swine produced by fumonisin $B_1$, a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostic Investigation, 2: 217-221.*

**Harvey R.B., Edrington T.S., Kubena L.F., Elissalde M.H., Rottinghaus G.E.** (1995). Influence of aflatoxin and fumonisin $B_1$-containing culture material on growing barrows. *American Journal of Veterinary Research, 56: 1668-1672.*

**Harvey R.B., Edrington T.S., Kubena L.F., Elissalde M.H., Casper H.H., Rottinghaus G.E., Turk J.R.** (1996). Effects of dietary fumonisin $B_1$-containing culture material, deoxynivalenol-contaminated wheat, or their combination on growing barrows. *American Journal of Veterinary Research, 57: 1790-1794.*

**Haschek W.M., Motelin G., Ness D.K., Harlin K.S., Hall W.F., Vesonder R.F., Peterson R.E., Beasley V.R.** (1992). Characterization of fumonisin toxicity in orally and intravenously dosed swine. *Mycopathologia, 117: 83-96.*

**Howard P.C., Eppley R.M., Stack M.E., Warbritton A., Voss K.A., Lorentzen R.J., Kovach R.M., Bucci T.J.** (2001a). Fumonisin $B_1$ carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. *Environmental Health Perspectives, 109: 277-282.*

**Howard P.C., Warbritton A., Voss K.A., Lorentzen R.J., Thurman J.D., Kovach R.M., Bucci T.J.** (2001b). Compensatory regeneration as a mechanism for renal tubule carcinogenesis of fumonisin $B_1$ in F344/N/Nctr BR rats. *Environmental Health Perspectives, 109: 309-314.*

**Jaskiewicz K., Marasas W.F.O., Taljaard J.J.** (1987). Hepatitis in vervet monkeys caused by *Fusarium moniliforme*. *Journal of Comparative Pathology, 97: 281-291.*

**Javed T., Richard J.L., Bennett G.A., Dombrink-Kurtzman M.A., Bunte R.M., Koelkebeck K.W., Côté L.M., Leeper R.W., Buck W.B.** (1993). Embryopathic and embryocidal effects of purified fumonisin $B_1$ or *Fusarium proliferatum* culture material extract on chicken embryos. *Mycopathologia, 123: 185-193.*

**Kellerman T.S., Marasas W.F.O., Thiel P.G., Gelderblom W.C., Cawood M., Coetzer J.A.** (1990). Leukoencephalomalacia in two horses induced by oral dosing of fumonisin $B_1$. *Onderstepoort Journal of Veterinary Research, 57: 269-275.*

**Langhout D.J., Spicer L.J., Geisert R.D.** (1991). Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. *Journal of Animal Science, 69: 3321-3334.*

**Malekinejad H., Schoevers E.J., Daemen I.J.J.M., Zijstra C., Colenbrander B., Fink-Gremmels J., Roelen B.A.J.** (2007). Exposure of

oocytes to the *Fusarium* toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. *Biology of Reproduction, 77: 840-847.*

**Marasas W.F.O., Kellerman T.S., Gelderblom W.C., Coetzer J.A., Thiel P.G., van der Lugt J.J.** (1988). Leukoencephalomalacia in a horse induced by fumonisin $B_1$ isolated from *Fusarium moniliforme. Onderstepoort Journal of Veterinary Research, 55: 197-203.*

**Marin D.E., Gouze M.E., Taranu I., Oswald I.P.** (2007). Fumonisin $B_1$ alters cell cycle progression and interleukin-2 synthesis in swine peripheral blood mononuclear cells. *Molecular Nutrition and Food Research, 51: 1406-1412.*

**Medvedova M., Kolesarova A., Capcarova M., Labuda R., Sirotkin A.V., Kovacik J., Bulla J.** (2011). The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells *in vitro. Journal of Environmental Science and Health - Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 46: 213-219.*

**Merrill A.H. Jr., Sullards M.C., Wang E., Voss K.A., Riley R.T.** (2001). Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives, 109: 283-289.*

**Minervini F., Lacalandra G.M., Filannino A., Garbetta A., Nicassio M., Dell'Aquila M.E., Visconti A.** (2010). Toxic effects induced by mycotoxin fumonisin $B_1$ on equine spermatozoa: assessment of viability, sperm chromatin structure stability, ROS production and motility. *Toxicology In Vitro, 24: 2072-2078.*

**Motelin G.K., Haschek W.M., Ness D.K., Hall W.F., Harlin K.S., Schaeffer D.J., Beasley V.R.** (1994). Temporal and dose-response features in swine fed corn screenings contaminated with fumonisin mycotoxins. *Mycopathologia, 126: 27-40.*

**Murphy P.A., Rice L.G., Ross P.F.** (1993). Fumonisin $B_1$, $B_2$ and $B_3$ content of Iowa, Wisconsin, and Illinois corn and corn screenings. *Journal of Agricultural and Food Chemistry, 41: 263-266.*

**Norred W.P.** (1993). Fumonisins – mycotoxins produced by *Fusarium moniliforme. Journal of Toxicology and Environmental Health, 38: 309-328.*

**Osweiler G.D., Ross P.F., Wilson T.M., Nelson P.E., Witte S.T., Carson T.L., Rice L.G., Nelson H.A.** (1992). Characterization of an epizootic of pulmonary edema in swine associated with fumonisin in corn screenings. *Journal of Veterinary Diagnostic Investigation, 4: 53-59.*

**Ott L.** (1977). Multiple comparisons. In: *An Introduction to Statistical Methods and Data Analysis. Duxbury Press, North Scituate, MA, pp. 384-388.*

**Placinta C.M., D'Mello C.P.F., MacDonald A.M.C.** (1999). A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Animal Feed Science and Technology, 78: 21-37.*

**Ranzenigo G., Caloni F., Cremonesi F., Aad P.Y., Spicer L.J.** (2008). Effects of *Fusarium* mycotoxins on steroid production by porcine granulosa cells. *Animal Reproduction Science, 107: 115-130.*

**Riley R.T., Voss K.A., Norred W.P., Bacon C.W., Meredith F.I., Sharma R.P.** (1999). Serine palmitoyltransferase inhibition reverses anti-proliferative effects of ceramide synthase inhibition in cultured renal cells and suppresses free sphingoid base accumulation in kidney of BALBc mice. *Environmental Toxicology and Pharmacology, 7: 109-118.*

**Ross P.F., Ledet A.E., Owens D.L., Rice L.G., Nelson H.A., Osweiler G.D., Wilson T.M.** (1993). Experimental equine leukoencephalomalacia, toxic hepatosis, and encephalopathy caused by corn naturally contaminated with fumonisins. *Journal of Veterinary Diagnostic Investigation, 5: 69-74.*

**SAS** (1999). The SAS System for Windows: Version 8. *SAS Institute Inc., Cary, NC.*

**Schoevers E.J., Fink-Gremmels J., Colenbrander B., Roelen B.A.** (2010). Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. *Theriogenology, 74: 968-978.*

**Smith G.W., Constable P.D., Tumbleson M.E., Rottinghaus G.E., Haschek W.M.** (1999). Sequence of cardio- vascular changes leading to pulmonary edema in swine fed culture material containing fumonisin. *American Journal of Veterinary Research, 60: 1292-1300.*

**Smith G.W.** (2012). Fumonisins. In: *Gupta R.C. (Ed.), Veterinary Toxicology: Basic and Clinical Principles (second edition), Elsevier Inc., pp. 1205-1219.*

**Spicer L.J., Alpizar E., Echternkamp S.E.** (1993). Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production *in vitro*. *Journal of Animal Science, 71: 1232-1241.*

**Spicer L.J., Chamberlain C.S.** (1998). Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF-1)-induced steroid production and on IGF-1 receptors in cultured bovine granulosa cells and thecal cells. *Endocrine, 9: 153-161.*

**Spicer L.J., Chamberlain C.S., Maciel S.M.** (2002). Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. *Domestic Animal Endocrinology, 22: 237-254.*

**Spicer L.J., Hammond J.M.** (1987). 2-Hydroxyestradiol modulates a facilitative action of catecholamines on porcine granulosa cells. *Endocrinology, 120: 2375-2382.*

**Spicer L.J., Hammond J.M.** (1989). Catecholestrogens inhibit proliferation and DNA synthesis of porcine granulosa cells *in vitro*: comparison with estradiol, 5α-dihydrotestosterone, gonadotropins and catecholamines. *Molecular and Cellular Endocrinology, 64: 119-126.*

**Spiegel S., Merrill A.H. Jr.** (1996). Sphingolipid metabolism and cell growth regulation. *The FASEB journal, 10: 1388-1397.*

**Sydenham E.W., Shephard G.S., Thiel P.G., Marasas W.F.O., Stockestrom S.** (1991). Fumonisin contamination of commercial corn-based human foodstuffs. *Journal of Agricultural and Food Chemistry, 39: 2014-2018.*

**Tiemann U., Tomek W., Schneider F., Vanselow J.** (2003). Effects of the mycotoxins α- and β-zearalenol on regulation of progesterone synthesis in cultured granulosa cells from porcine ovaries. *Reproductive Toxicology, 17: 673-681.*

**Voss K.A., Chamberlain W.J., Bacon C.W., Norred W.P.** (1993). A preliminary investigation on renal and hepatic toxicity in rats fed purified fumonisin $B_1$. *Natural Toxins, 1: 222-228.*

**Voss K.A., Norred W.P., Plattner R.D., Bacon C.W.** (1989). Hepatotoxicity and renal toxicity in rats of corn samples associated with field cases of equine leukoencephalomalacia. *Food and Chemical Toxicology, 27: 89-96.*

**Voss K.A., Smith G.W., Haschek W.M.** (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. *Animal Feed Science and Technology, 137: 299-325.*

**Wang E., Norred W.P., Bacon C.W., Riley R.T., Merrill A.H. Jr.** (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *The Journal of Biological Chemistry, 266: 14486-14490.*

**Yoo H.S., Norred W.P., Showker J., Riley R.T.** (1996). Elevated sphingoid bases and complex sphingolipid depletion as contributing factors in fumonisin-induced cytotoxicity. *Toxicology and Applied Pharmacology, 138: 211-218.*

**Yoo H.S., Norred W.P., Wang E., Merrill A.H. Jr., Riley R.T.** (1992). Fumonisin inhibition of *de novo* sphingolipid biosynthesis and cytotoxicity are correlated in LLC-PK1 cells. *Toxicology and Applied Pharmacology, 114: 9-15.*

**Zacharias C., van Echten-Deckert G., Wang E., Merrill A.H. Jr., Sandhoff, K.** (1996). The effect of fumonisin $B_1$ on developing chick embryos: correlation between *de novo* sphingolipid biosynthesis and gross morphological changes. *Glycoconjugate Journal, 13: 167-175.*

**Zhu L., Yuan H., Guo C., Lu Y., Deng S., Yang Y., Wei Q., Wen L., He Z.** (2012). Zearalenone induces apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9-dependent mitochondrial signaling pathway. *Journal of Cellular Physiology, 227: 1814-1820.*

# CHAPTER 4

# Effects of fumonisin $B_1$ alone and combined with deoxynivalenol or zearalenone on steroid production by porcine granulosa cells

# 4. Effects of fumonisin $B_1$ alone and combined with deoxynivalenol or zearalenone on steroid production by porcine granulosa cells

## 4.1. Abstract

Fumonisin $B_1$ ($FB_1$) is a mycotoxin primarily produced by *Fusarium verticillioides* and *Fusarium proliferatum*, frequently occurring in corn in combination with trichothecenes and zearalenone. There is a lack of information on the potential effects of $FB_1$ on reproduction in swine. Thus, the goal of this study was to determine if $FB_1$, alone and combined with deoxynivalenol (DON) or α-zearalenol (α-ZOL), zearalenone major active metabolite, can impair swine reproductive functions via affecting granulosa cell steroidogenesis and gene expression. Granulosa cells from small porcine follicles (1-5 mm) were cultured for 2 days in 5% fetal bovine serum and 5% porcine serum-containing medium followed by 1 or 2 days in serum-free medium with or without added treatments. All doses of $FB_1$ (i.e., 0.01, 0.4, 10 and 14 μM) had no significant effect on estradiol production, whereas $FB_1$ showed a stimulatory effect on progesterone production induced by FSH plus insulin-like growth factor-I (IGF-I) at 10 and 14 μM. α-ZOL (9.4 μM) increased ($P < 0.0001$) FSH plus IGF-I-induced progesterone production by 51%. Combination of $FB_1$ with α-ZOL resulted in an increase of progesterone production (91%; $P < 0.0001$) that was significantly higher than that induced by either *Fusarium* mycotoxin alone. DON drastically inhibited (by 74%; $P < 0.0001$) progesterone production, and $FB_1$ had little effect on this response. α-ZOL had no effect on estradiol production, whereas decreased ($P < 0.05$) estradiol production when co-treated with $FB_1$. DON (3.4 μM) strongly inhibited (by 67%; $P < 0.0001$) estradiol production and no difference was detected in combination with $FB_1$ (10 μM). $FB_1$ (10 μM) decreased (by 23%; $P < 0.0001$) granulosa cell CYP11A1 mRNA abundance induced by FSH plus IGF-I but had no effect ($P > 0.10$) on basal or FSH plus IGF-I-induced granulosa cell CYP19A1 mRNA abundance. In conclusion, the present study indicates that $FB_1$ may alter steroid production in swine, and provides the first information on the interactions between $FB_1$ and DON or α-ZOL in granulosa cells. These interactions and direct ovarian effects should be considered in swine reproductive failures.

## 4.2. Introduction

Fumonisins, zearalenone and trichothecenes, such as deoxynivalenol (DON) and T-2 toxin, are the major *Fusarium* mycotoxins occurring throughout the world in cereal grains and animal feeds. Exposure to these mycotoxins has been positively linked to reproductive disorders in pigs (Alm et al., 2002, 2006; Malekinejad et al., 2007; Gbore and Egbunikem, 2008; Ranzenigo et al., 2008; Caloni et al., 2009; Gbore, 2009a, 2009b; Schoevers et al., 2010).

Fumonisins are a family of mycotoxins produced by a limited number of *Fusarium* species, of which *Fusarium verticillioides* and *Fusarium proliferatum* are the most frequent worldwide (Marin et al., 2004). Fumonisins occur particularly in corn and corn-based products and, co-occurrence with other *Fusarium* mycotoxins, such as zearalenone and DON, is regularly observed (EFSA, 2005). Fumonisin $B_1$ ($FB_1$) is the most significant of this family in terms of toxicity and occurrence (EFSA, 2005). Recent surveys have raised concerns about the extent of $FB_1$ contamination and its implication for animal health and productivity (Boutigny et al., 2012; Garrido et al., 2012; Rodrigues and Naehrer, 2012). $FB_1$ induces different species-specific effects in domestic and laboratory animals including pulmonary edema in pigs (Harrison et al., 1990), leukoencephalomalacia in horses (Kellerman et al., 1990), hepatocarcinoma in rats (Gelderblom et al., 1991) and mice (Howard et al., 2001a), nephrocarcinoma in rats (Howard et al., 2001b), and nephropathy in rats (Voss et al., 1993) and rabbits (Gumbrecht et al., 1995). Moreover, $FB_1$ was found to cause adverse reproductive and developmental effects in rats (Flynn et al., 1996; Gbore et al., 2012), Syrian hamsters (Floss et al., 1994), chickens (Bacon et al., 1995; Javed et al., 1993) and rabbits (Ogunlade et al., 2006; Ewuola and Egbunike, 2010). Reproductive inefficiency is recognized as the most costly limiting constraint to efficient animal production (Gbore, 2009a; Ewuola and Egbunike, 2010). However, studies on reproductive toxicity induced by $FB_1$ in livestock are rare. Recently, dietary $FB_1$ has been reported to delay attainment of sexual maturity in growing pigs (Gbore, 2009a) and to reduce sperm production and semen quality in boars, with potential to impair fertility (Gbore and Egbunikem, 2008; Gbore, 2009b). Moreover, $FB_1$ was found to affect *in vitro* some functional parameters of equine spermatozoa, such as sperm chromatin stability and motility (Minervini et al., 2010). Because reproduction is the bedrock of animal production (Gbore, 2009a; Ewuola and Egbunike, 2010), further studies are needed to assess the potential of $FB_1$ to influence reproductive function in livestock. Previous *in vitro* studies demonstrated that other *Fusarium* mycotoxins such as zearalenone, DON and T-2 toxin may alter granulosa cell function in swine (Tiemann et al., 2003; Ranzenigo et al., 2008; Caloni et al., 2009; Medvedova et al., 2011; Zhu et al., 2012), which is crucial in the process of normal folliculogenesis and oocyte

growth and development (Petro et al., 2012). Thus, the goal of this study was to determine if $FB_1$, alone or combined with other *Fusarium* mycotoxins such as DON or zearalenone, can negatively impact swine reproductive function *via* affecting granulosa cell steroidogenesis and gene expression.

## 4.3. Materials and methods

### *4.3.1. Reagents and hormones*

The following reagents and hormones were used for cell culture: Dulbecco modified Eagle medium (DMEM), Ham's F-12, gentamicin, L-glutamine, sodium bicarbonate, trypan blue, collagenase, DNase, porcine serum (PS), and fetal calf serum (FCS) all obtained from Sigma-Aldrich Chemical Company (St. Louis, MO); ovine follicle-stimulating hormone (FSH; 175 x NIH-FSH-S1 U/mg) from Dr. A. F. Parlow, National Hormone & Pituitary Program (Torrance, CA); recombinant human insulin-like growth factor-I (IGF-I) and recombinant human fibroblast growth factor-9 (FGF9) from R&D Systems (Minneapolis, MN) and testosterone from Steraloids (Wilton, NH).
The reagents used in RNA extraction were: TRIzol® Reagent and DEPC-treated water from Life Technologies, Inc. (Gaithersburg, MD), chloroform from Sigma-Aldrich Chemical Company (St. Louis, MO), isopropyl alcohol and ethanol from Pharmco Products Inc. (Brookfield, CT).
The reagents used for radioimmunoassays were: [$^{125}$I]iodo-progesterone (ICN Biomedicals, Irvine, CA), anti-progesterone rabbit antiserum (X-16) provided by Dr. P. Natashima Rao (Southwestern Foundation for Research Education, San Antonio, TX), normal rabbit serum (NRS) (Linco Research, Inc., St. Charles, MO), $^{125}$I-estradiol (ICN Biomedicals, Costa Mesa, CA), anti-estradiol rabbit antibody (Lilly Research Laboratories, Indianapolis, IN), and goat anti-rabbit antibody (Linco Research, Inc., St. Charles, MO).

### *4.3.2. Cell culture*

Ovaries from non-pregnant gilts were collected from a local slaughterhouse and were treated as previously described (Spicer and Hammond, 1987, 1989; Ranzenigo et al., 2008). The ovaries were washed three times in 0.9% saline, immersed in 70% ethanol for 30 s, washed again three times with 0.9% saline and transported to the laboratory in 0.9% saline with 1% streptomycin/penicillin on ice. Follicular fluid was aspirated aseptically from small follicles (1-5 mm) with a 25-gauge needle (Figure 4.1) and granulosa cells were isolated and re-suspended in 2 mL of serum-free medium containing collagenase and DNase at

1.25 mg/mL and 0.5 mg/mL, respectively, to prevent clumping of cells as previously described (Spicer et al., 2002). Viability of porcine granulosa cells from small follicles was determined by trypan blue exclusion method (Langhout et al., 1991; Spicer et al., 1993; Tiemann et al., 2003) and averaged 32 ± 2.1%.

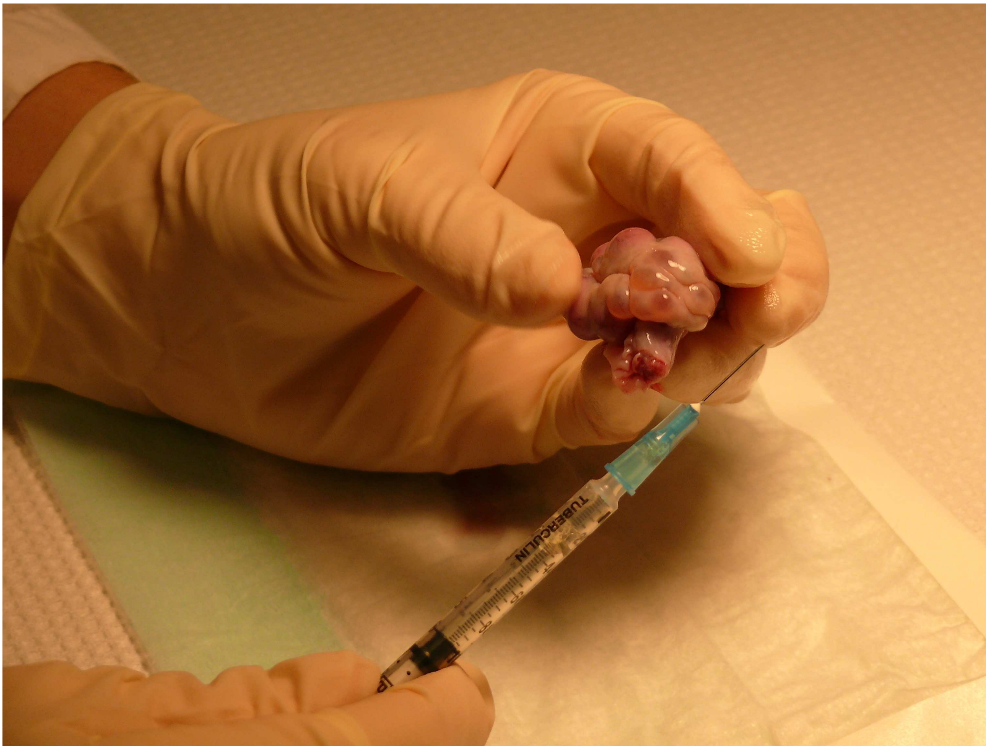



**Figure 4.1.** Follicular fluid aspiration. Follicular fluid was aspirated aseptically from small follicles (1-5 mm) with a 25-gauge needle.

Approximately 3 x $10^5$ viable cells were plated on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ) in 1 mL of medium composed of a mixture of 1:1 DMEM and Ham's F-12 containing glutamine (2 mM), gentamicin (0.12 mM) and sodium bicarbonate (38.5 mM) (Figure 4.2).
Cultures were kept at 38.5° C in a humidified 95% air-5% $CO_2$ environment, and medium was changed every 24 h (Figure 4.3). To obtain an optimal attachment, cells were maintained in the presence of 5% FCS and 5% PS for the first 48 h of culture. Cells were then washed twice with 0.5 mL of serum-free medium and incubations continued in serum-free medium for 1 or 2 days containing 500 ng/mL of testosterone (as an estradiol precursor; Spicer and Chamberlain, 1998; Ranzenigo et al., 2008) with or without added treatments as described below.

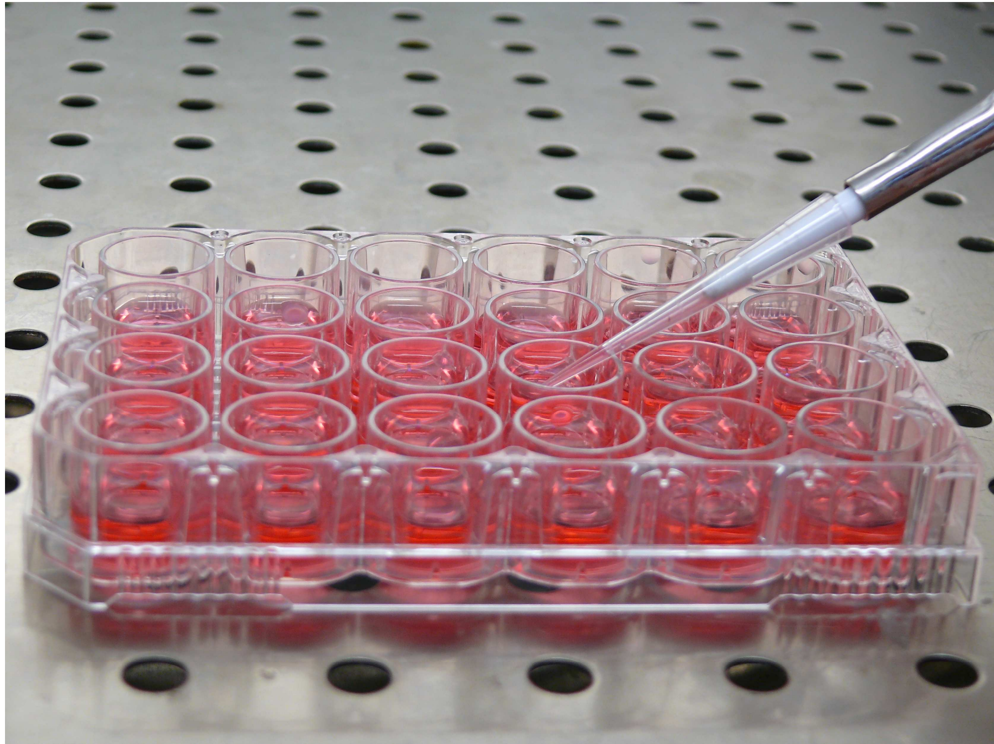


**Figure 4.2.** Cell seeding. Approximately 3 x $10^5$ viable cells in 10 to 25 μL of medium were added to Falcon multiwell plates containing 1 mL of medium.

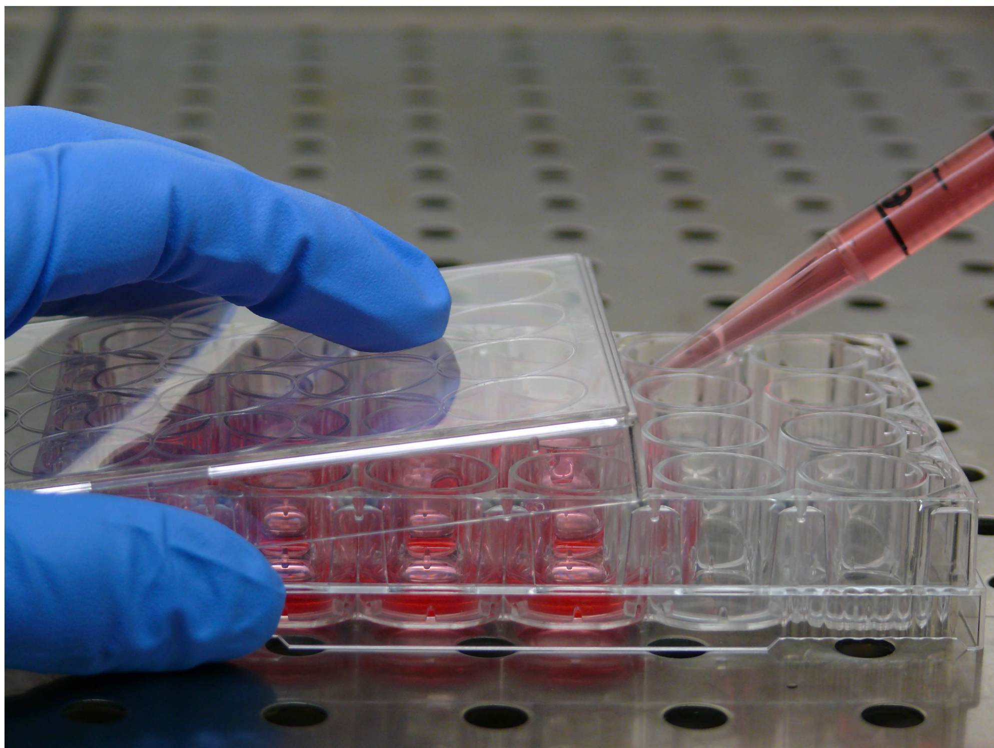


**Figure 4.3.** Daily medium change.

### *4.3.3. Determination of steroid concentrations*

Medium was collected from individual wells and frozen at -20 ºC for subsequent steroid analyses. Concentrations of progesterone and estradiol in culture medium were determined by radioimmunoassays (RIA) as previously described (Spicer and Hammond, 1987; Spicer et al., 1990; Langhout et al., 1991). Numbers of granulosa cells in the same wells from which medium was collected were determined by a Coulter counter (model Z2; Beckman Coulter, Inc., Hialeah, FL), and used to calculate steroid production on a ng or pg per $10^5$ cell basis. Briefly, cells were gently washed twice with 0.9% saline solution (500 µL), exposed to 500 µL of trypsin (0.25% wt/vol=2.5 mg/mL) for 20 min at room temperature, and then scraped from each well and enumerated as previously described (Spicer and Hammond, 1987; Langhout et al., 1991; Ranzenigo et al., 2008). Cell aggregates were minimized by pipetting cell suspensions back and forth through a 500 µL pipette tip three to five times.

Progesterone RIA

Progesterone RIA were conducted using rabbit antiserum (X-16), which serves as the first antibody (diluted 1:3000 with assay buffer: PBS, EDTA, $NaN_3$, and gelatin), raised against BSA-11 glutamate derivative as described by Baraño and Hammond (1985). Goat anti-rabbit antibody (diluted 1:15 with assay buffer) was used as the second antibody (Linco Research, Inc., St. Charles, MO). [$^{125}$I]Iodo-progesterone (ICN Biomedicals, Costa Mesa, CA) was used as the tracer. A progesterone standard curve was prepared from a stock concentration of 80.0 ng/mL that was serially diluted with assay buffer to concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.625, 0.31, and 0.16 ng/mL. In duplicate, 20 µL to 100 µL of medium samples were combined with the appropriate volume of assay buffer to make a total volume of 100 µL. One hundred µL of tracer and first antibody were added and all samples were mixed and allowed to incubate at 37 °C for 1 h. Following incubation, 200 µL of second antibody were added and all samples were incubated overnight at 4 °C. The following day, 50 µL of normal rabbit serum (NRS) (diluted 1:5 with assay buffer from a 15% NRS stock) were added to all samples. Samples were centrifuged at 4 °C in a Sorvall Model RC-3 (Thermo Fisher Scientific, Inc., Miami, OK) at 1800 x *g* for 25 min. Supernatant was aspirated and precipitates were counted for 1 min using a Cobra AII Auto-Gamma counter (Packard Instrument Co., Downers Grove, IL). The intra- and interassay coefficients of variation were 7 and 13%, respectively, for the progesterone RIA.

Estradiol RIA

Estradiol RIA were conducted using anti-estradiol rabbit antibody (diluted 1:12 with assay buffer), which serves as the first antibody (Lilly Research Laboratories, Indianapolis, IN), and goat anti-rabbit antibody (diluted 1:15 with assay buffer) which serves as the second antibody (Linco Research, Inc., St. Charles, MO). Radiolabled estradiol ($^{125}$I-estradiol) was used as the tracer (ICN Biomedicals, Costa Mesa, CA). The assay buffer was the same as the progesterone RIA buffer described above. An estradiol dose response curve was prepared from a stock concentration of 256 pg/100 µL that was serially diluted to 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 pg/100 µL using assay buffer. In duplicate, sample media was added at either 50 or 100 µL and (if needed) combined with assay buffer to make a total volume of 100 µL. Two hundred µL of tracer were added to all samples, along with 100 µL of first antibody. All tubes were mixed and allowed to incubate for 1 h at 37 °C. Following this incubation, 200 µL of second antibody were added and the assay allowed to incubate at 4 °C overnight. The following day, assay tubes were centrifuged, supernatant aspirated, and precipitate counted as described for the progesterone RIA. The intra- and interassay coefficients of variation were 8% and 17%, respectively, for the estradiol RIA.

### *4.3.4. RNA analysis*

RNA extraction

At the end of the treatment period, cells from two replicate wells were lysed in 500 µL of TRIzol® reagent and RNA was extracted as previously described (Voge et al., 2004; Aad et al., 2006). Briefly, 250 µL TRIzol® reagent was added to all wells and cells were lysed by repeated pipetting and then combined with their respective replicates. Combined wells were then transferred to 1.5 mL eppendorf tubes. Each treatment containing 4 wells generated 2 replicate samples of RNA. Cell lysates were incubated in TRIzol® reagent for approximately 5 min at room temperature, then 100 µL of chloroform was added to each sample followed by a 15 s vortex. After approximately a 2 min incubation at room temperature, samples were centrifuged at 3500 x *g* for 30 min at 4 °C using eppendorf centrifuge 5417C (Brinkmann Instruments, Westbury, NY). The upper aqueous phase of each sample was then transferred to a fresh eppendorf tube and RNA was precipitated using 250 µL isopropanol. Samples were incubated at room temperature for 10 min and then centrifuged at 3500 x *g* for 10 min at 4 °C. The RNA pellets were washed after discarding the supernatant with 500 µL of 70% ethanol and allowed to dry at room temperature. The RNA pellets were suspended in 16.5 µL of DEPC-treated water. RNA was quantitated by spectrophotmetry at 260 nm using a NanoDrop

ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Aliquots of 1.5 μL of RNA were used to determine the concentration in ng/μL as well as the purity given as a ratio of 260/280 nm where values between 1.8 and 2.2 were acceptable. RNA was then diluted to 10 ng/μL in DEPC-treated water and stored at -80 °C until used for quantification of target gene expression. Just prior to use, an RNA aliquot was thawed on ice for 3-5 min.

<u>Quantitative RT-PCR</u>

The target gene primers (forward, reverse) and probe sequences for aromatase enzyme (CYP19A1; Accession U92245) were TGCCAAGAATGTTCCT TACAGGTA, CAGAGTGACCTTCATCATGACCAT and CATTTGGCTTT GGGCCCCGG, respectively; and for P450 side-chain cleavage enzyme (CYP11A1; Accession NM 214427) were CTCCGTGACCCTGCAGAGATAC, ATAGACGGCCACTTGTACCAATG and TTGGTTCTTCGAGATTACA TGATTCCTGCC, respectively.

A BLAST search (www.ncbi.nlm.nih.gov/BLAST) was also conducted to insure the specificity of the designed primers and probe and to assure that they were not designed from any homologous regions coding for other genes. The differential expression of target gene mRNA granulosa cells was quantified using the one-step multiplex real-time RT-PCR reaction for Taqman® Gold RT-PCR Kit (Applied Biosystems, Foster City, CA) as previously described (Spicer and Aad, 2007; Ranzenigo et al., 2008). Briefly, based on preliminary optimization results, 100 ng of total RNA was amplified in a total reaction volume of 25 μL consisting of 200 nM forward primer, 200 nM reverse primer and 100 nM fluorescent (FAM/TAMRA) probe for each target gene, 50 nM of 18S rRNA primers and 100 nM of the 18S rRNA VIC-labeled probe, along with 12.5 μL of TaqMan Master Mix without uracil N-glycosylase, and 1 U Multiscribe with RNase inhibitor (Applied Biosystems). Thermal cycling conditions were set to 30 min at 50 ºC for reverse transcription, 95 ºC for 10 min for AmpliTaq Gold activation, and finished with 55 cycles at 95 ºC for 15 s for denaturing and 60 ºC for 1 min for annealing and extension. All samples were run in duplicate. The 18S rRNA values were used as internal controls to normalize samples for any variation in amounts of RNA loaded, and relative quantification of target gene mRNAs was expressed using the comparative threshold cycle method as previously described (Voge et al., 2004; Aad et al., 2006). Briefly, the ΔCt was determined by subtracting the 18S Ct value from the target unknown value. For each target gene, the ΔΔCt was determined by subtracting the higher ΔCt (the least expressed unknown) from all other ΔCt values. Fold changes in target gene mRNA abundance were calculated as being equal to $2^{-\Delta\Delta Ct}$.

### *4.3.5. Experimental design*

Experiment 1 was designed to determine the effects of different doses of $FB_1$ (i.e., 0.01, 0.4 and 14 μM) on steroid production in the presence of FSH with or without IGF-I. Cells were cultured for 48 h in 5% FCS and 5% PS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing FSH (30 ng/mL), IGF-I (0 or 30 ng/mL) with or without the various doses of $FB_1$ (i.e., 0.01, 0.4 and 14 μM). After 48 h of treatment, medium was collected for progesterone and estradiol determinations and cells were counted.
Experiment 2 was designed to evaluate the effects of DON and α-ZOL with and without $FB_1$ on FSH plus IGF-I-induced steroid production. Cells were cultured for 48 h in 5% FCS and 5% PS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing 30 ng/mL of IGF-I and 30 ng/mL of FSH with or without $FB_1$ (10 μM), DON (3.4 μM) and α-ZOL (9.4 μM). After 48 h of treatment, medium was collected for progesterone and estradiol determinations and cells were counted.
Experiment 3 was designed to determine the effect of $FB_1$ (10 μM) on FSH plus IGF-I-induced CYP11A1 and CYP19A1 mRNA abundance in granulosa cells from small porcine follicles. Cells were cultured for 48 h in 5% FCS and 5% PS, washed twice with serum-free medium as described earlier, and cells treated for 24 h in serum-free medium containing 30 ng/mL of IGF-I and 30 ng/mL of FSH with or without $FB_1$ (10 μM). After 24 h of treatment, medium was aspirated and cells were lysed for RNA extraction.
Experiment 4 was conducted to determine if inhibition of *de novo* cholesterol synthesis using simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, altered the $FB_1$ response. Cells were cultured for 48 h in 5% FCS and 5% PS, washed twice with serum-free medium as described earlier, and cells treated for 24 h in serum-free medium containing simvastatin (0 or 3 μg/mL), IGF-I (30 ng/mL) and FSH (30 ng/mL) with or without $FB_1$ (10 μM). After 48 h of treatment, medium was collected for progesterone determinations and cells were counted.

### *4.3.6. Statistical analysis*

Experimental data are presented as the least squares means ± SEM of measurements from replicated culture wells. Each experiment was performed three times with different pools of granulosa cells collected from two to six ovaries for each pool and each treatment replicated three times within each experiment. For mRNA experiments, treatments were applied in quadruplicate culture wells with each mRNA sample being obtained from two wells. Treatment

effects and interactions were assessed using the GLM procedure of the Statistical Analysis System (SAS, 1999). Main effects were treatment, experiment and their interaction when data from more than one experiment were analyzed. Steroid production was expressed as ng or pg/$10^5$ cells per 24 h, and cell numbers determined at the end of the experiment were used for this calculation. Mean differences in steroid production and mRNA abundance between treatments were determined using the Fisher's protected least significant difference (LSD) procedure (Ott, 1977).

## 4.4. Results

### *4.4.1. Experiment 1: dose-response of $FB_1$ on steroid production in the presence of FSH with or without IGF-I*

Experiment 1 was conducted to determine the effects of different doses of $FB_1$ on steroid production in the presence of FSH with or without IGF-I. In the absence of IGF-I, all doses of $FB_1$ (i.e., 0.01, 0.4 and 14 μM) had no significant effect ($P > 0.10$) on progesterone production (Figure 4.4A). In the presence of FSH plus IGF-I, at 14 μM $FB_1$ increased ($P < 0.0001$) progesterone production by 42%, whereas progesterone production was not affected by 0.01 μM and 0.4 μM of $FB_1$ (Figure 4.4A). All doses of $FB_1$ had no effect ($P > 0.10$) on estradiol production in the presence of FSH either with or without IGF-I (Figure 4.4B).

### *4.4.2. Experiment 2: interaction between $FB_1$ and DON or a-ZOL on FSH plus IGF-I-induced steroid production*

Experiment 2 was conducted to determine the effects of DON and α-ZOL with and without $FB_1$ on FSH plus IGF-I-induced steroid production. Alone, $FB_1$ or α-ZOL increased ($P < 0.0001$) FSH plus IGF-I-induced progesterone production by 59% and 51%, respectively (Figure 4.5A). Combination of $FB_1$ with α-ZOL resulted in an increase of progesterone production (91%; $P < 0.0001$) that was significantly higher than that induced by either *Fusarium* mycotoxin alone (Figure 4.5A). DON drastically inhibited (by 74%; $P < 0.0001$) progesterone production, and $FB_1$ had little effect on this response (Figure 4.5A). $FB_1$ and α-ZOL alone had no effect ($P > 0.10$) on FSH plus IGF-I induced estradiol production, whereas DON strongly inhibited (by 67%; $P < 0.0001$) estradiol production (Figure 4.5B). $FB_1$ did not influence the effect of DON on estradiol production, whereas α-ZOL decreased ($P < 0.05$) estradiol production when co-treated with $FB_1$ (Figure 4.5B).

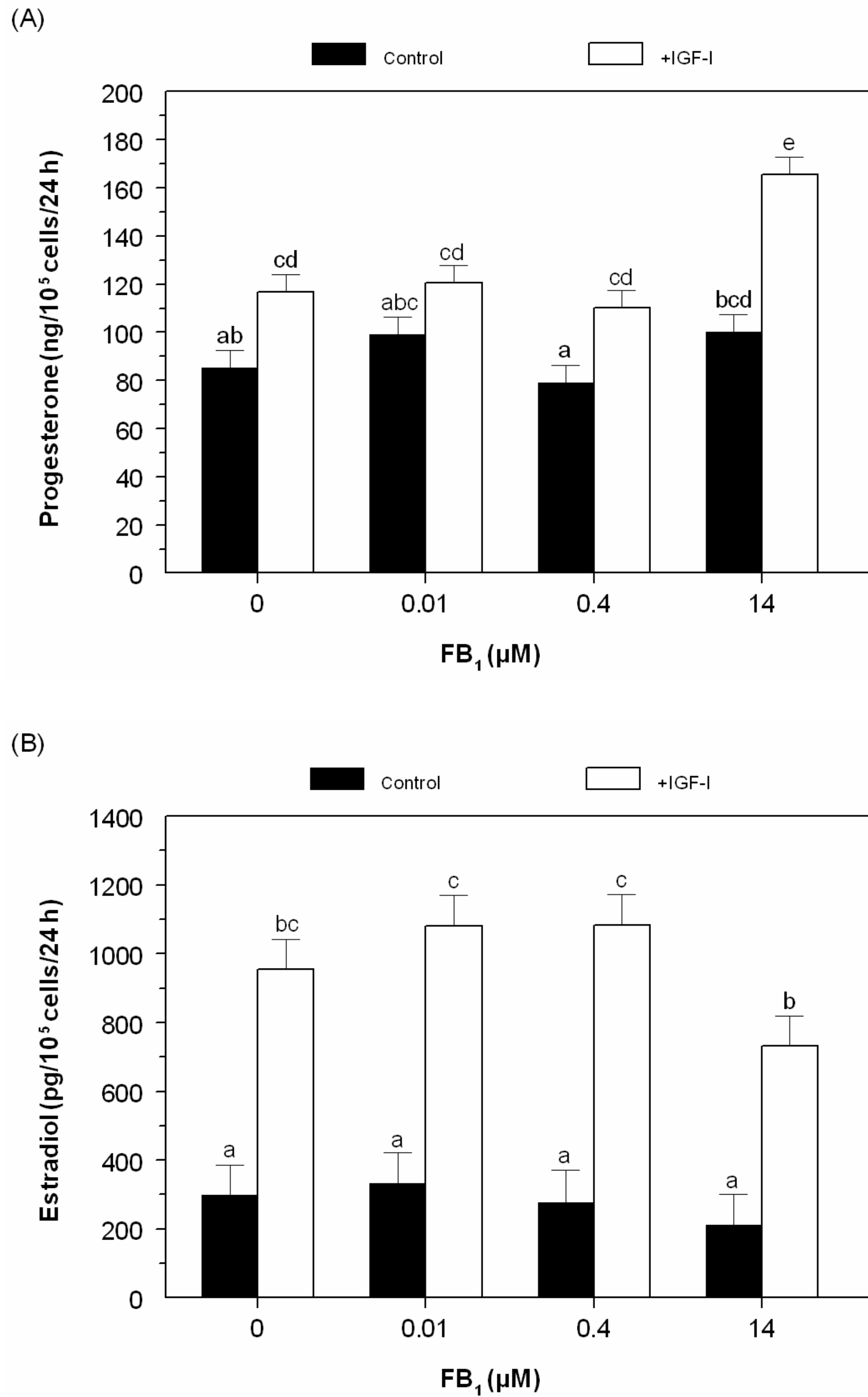



**Figure 4.4.** Effect of $FB_1$ on progesterone (Panel A) and estradiol (Panel B) production by granulosa cells from porcine follicles (Experiment 1). Granulosa cells were cultured for 4 days as described in Materials and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF-I (0 or 30 ng/mL) with or without the various doses of $FB_1$. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-e) differ ($P < 0.05$).

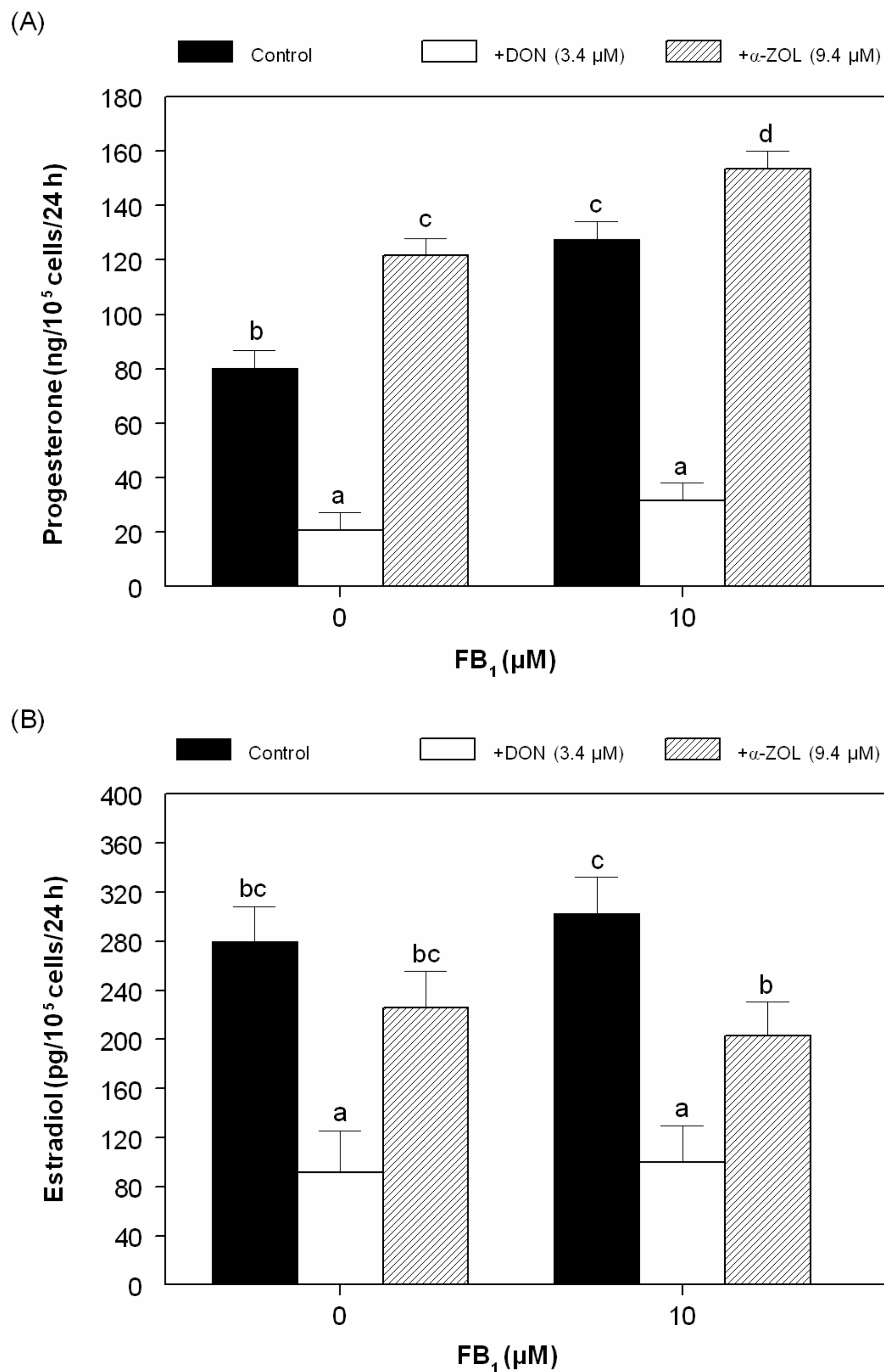



**Figure 4.5.** Interaction between $FB_1$ and deoxynivalenol (DON) or α-zearalenol (α-ZOL) on FSH plus IGF-I-induced progesterone (Panel A) and estradiol (Panel B) production by granulosa cells from porcine follicles (Experiment 2). Granulosa cells were cultured for 4 days as described in Materials and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing 30 ng/mL of IGF-I and 30 ng/mL of FSH with or without $FB_1$ (10 μM), DON (3.4 μM) and α-ZOL (9.4 μM). Values are means from three separate experiments ($n$ = 9). Means without a common letter (a-d) differ ($P$ < 0.05).

### 4.4.3. *Experiment 3: $FB_1$ effects on granulosa cell CYP11A1 and CYP19A1 mRNA*

Experiment 3 was carried out to determine the effect of $FB_1$ on CYP11A1 and CYP19A1 mRNA abundance. $FB_1$ (10 μM) had no effect ($P > 0.10$) on basal granulosa cell CYP11A1 mRNA concentrations; whereas $FB_1$ (10 μM) decreased (by 23%; $P < 0.0001$) granulosa cell CYP11A1 mRNA abundance induced by FSH plus IGF-I (Figure 4.6). $FB_1$ (10 μM) had no effect ($P > 0.10$) on basal or FSH plus IGF-I-induced granulosa cell CYP19A1 mRNA abundance (Figure 4.7).

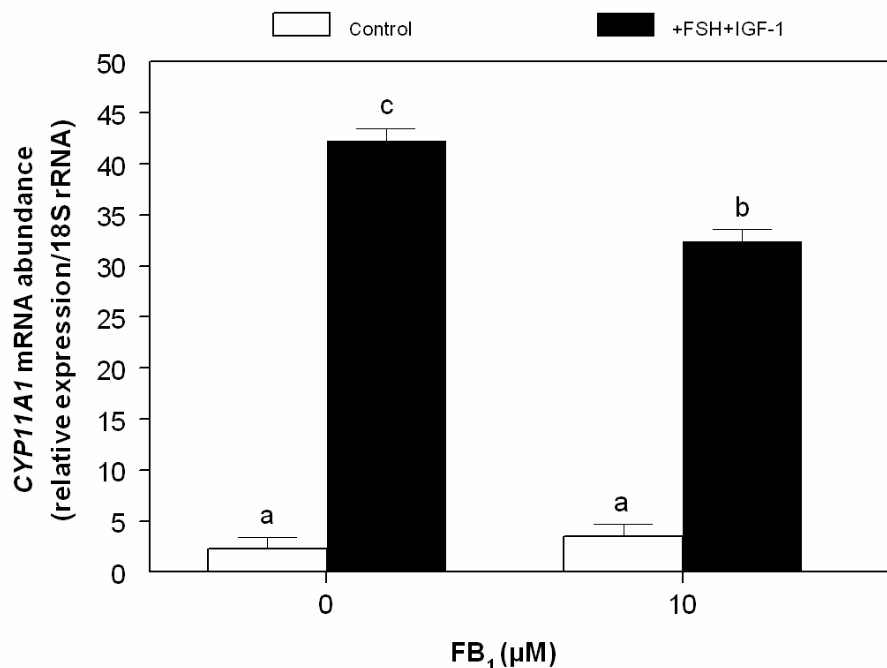



**Figure 4.6.** Effect of $FB_1$ on CYP11A1 mRNA abundance in granulosa cells from porcine follicles (Experiment 3). Granulosa cells were cultured for 3 days as described in Materials and Methods. During the last day of culture, cells were treated in serum-free medium with no additions (Control) or 30 ng/mL of FSH and IGF-I with or without $FB_1$ (10 μM). Values are means from three separate experiments ($n = 6$). Means without a common letter (a-d) differ ($P < 0.05$).

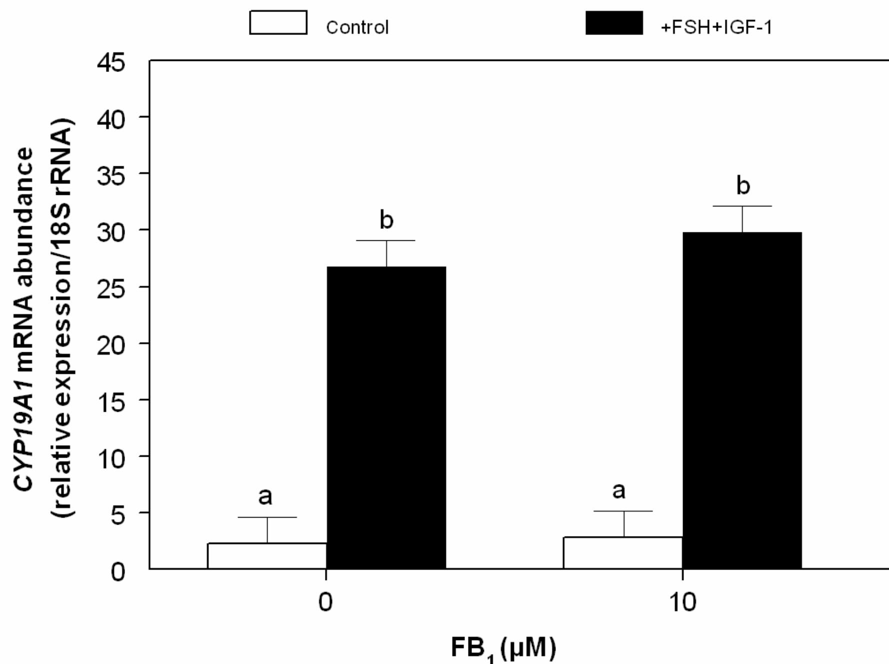



**Figure 4.7.** Effect of $FB_1$ on CYP19A1 mRNA abundance in granulosa cells from porcine follicles (Experiment 3). Granulosa cells were cultured for 3 days as described in Materials and Methods. During the last day of culture, cells were treated in serum-free medium with no additions (Control) or 30 ng/mL of FSH and IGF-I with or without $FB_1$ (10 μM). Values are means from three separate experiments (*n* = 6). Means without a common letter (a-d) differ ($P < 0.05$).

### *4.4.4. Experiment 4: effect of simvastatin on the stimulatory effect of $FB_1$ on FSH plus IGF-I-induced progesterone production*

Experiment 4 was conducted to determine if inhibition of *de novo* cholesterol synthesis using simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, altered the $FB_1$ response. In the presence of FSH plus IGF-I, simvastatin reduced ($P < 0.0001$) progesterone production by 51% (Figure 4.8). $FB_1$ (10 μM) increased ($P < 0.0001$) progesterone production by 59% and 56% in the absence and presence of simvastatin, respectively (Figure 4.8).

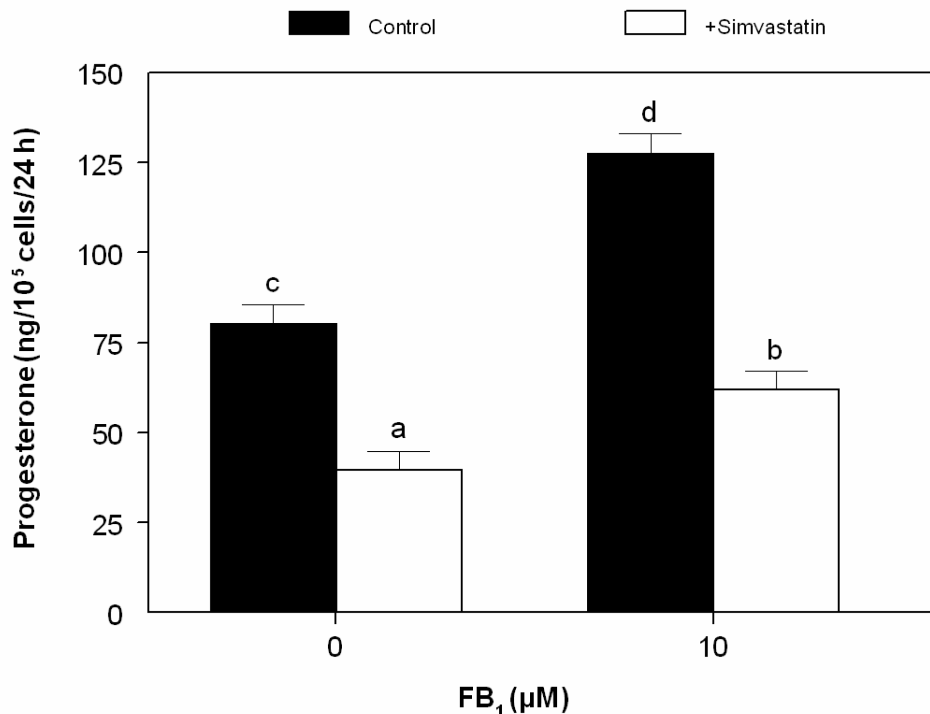



**Figure 4.8.** Effect of simvastatin on stimulatory effect of $FB_1$ on progesterone production by granulosa cells from porcine follicles (Experiment 4). Granulosa cells were cultured for 4 days as described in Materials and Methods. During the last 2 days of culture, cells were treated in serum-free medium with simvastatin (0 or 3 μg/mL), IGF-I (30 ng/mL) and FSH (30 ng/mL) with or without $FB_1$ (10 μM). Values are means from three separate experiments ($n$ = 9). Within a panel, means without a common letter (a-d) differ ($P < 0.05$).

## 4.5. Discussion

Despite recent surveys that have raised concerns about the extent of $FB_1$ contamination (Boutigny et al., 2012; Garrido et al., 2012; Rodrigues and Naehrer, 2012), data on reproductive effects of $FB_1$ are scarce. So far, effects of $FB_1$ on granulosa cell function, which is crucial in the process of normal folliculogenesis and oocyte growth and development (Petro et al., 2012), have not been previously investigated in any species. In the present study porcine granulosa cells were used to test the toxic potential of $FB_1$ on reproduction in swine. The results revealed that: (1) $FB_1$ increased progesterone production induced by FSH and IGF-I whereas had no effect on estradiol production; (2) DON strongly inhibited FSH plus IGF-I-induced progesterone and estradiol production and $FB_1$ had no significant effect on this response; (3) α-ZOL increased FSH plus IGF-I-induced progesterone production and concomitant treatment with $FB_1$ resulted in an increase of progesterone production that was

significantly higher than that induced by either *Fusarium* mycotoxin alone; (4) α-ZOL had no effect on FSH plus IGF-I-induced estradiol production, whereas decreased estradiol production when co-treated with $FB_1$; and (5) $FB_1$ decreased granulosa cell CYP11A1 mRNA abundance induced by FSH plus IGF-I whereas had no effect on gene expression of CYP19A1.

A stimulatory effect of $FB_1$ was observed on granulosa cell progesterone production induced by FSH and IGF-I at doses $\geq$ 10 μM. Progesterone is an essential regulator of the reproductive events and plays key roles in ovulation, zygote implantation and the subsequent maintenance of pregnancy (Graham and Clarke, 1997). The accumulation of progesterone may result from an increased activity of the first enzyme of the steroidogenic pathway, the mitochondrial enzyme cytochrome P450scc (CYP11A1), which catalyzes the conversion of cholesterol to pregnenolone (Lahav et al., 1996). This conversion is the rate-limiting step and principal site of hormonal regulation of progesterone biosynthesis (Hsueh et al., 1984). Therefore, to understand the mechanism by which $FB_1$ increases progesterone production, the effect of $FB_1$ on the expression of CYP11A1 in granulosa cells was evaluated. $FB_1$ was found to decrease CYP11A1 mRNA abundance induced by FSH and IGF-I suggesting that $FB_1$ increases progesterone production through a different mechanism.
Previous studies have shown that porcine granulosa cells cultured in serum-free medium obtain the cholesterol needed for steroidogenesis through *de novo* cholesterol synthesis (Baraño and Hammond, 1986; Spicer et al., 1990). The rate-limiting enzyme in cholesterol biosynthesis is HMG-CoA reductase. Our study with simvastatin, an inhibitor of HMG-CoA reductase, indicates that the stimulatory effect of $FB_1$ on progesterone production is not dependent on HMG-CoA reductase activity as $FB_1$ was found to increase progesterone production approximately to the same extent in the absence and presence of simvastatin (59% and 56%, respectively).
Taken together, these results suggest that $FB_1$-induced progesterone synthesis in granulosa cells could be caused by increased activity of the microsomal enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD), which catalyzed the conversion of pregnenolone to progesterone, and/or increased expression of ovarian steroidogenic acute regulatory (StAR) protein. This protein mediates the movement of free cholesterol from the outer mitochondrial membrane to the inner, where P450scc resides. The delivery of substrate cholesterol is the rate-limiting step in all steroid production (Lucki and Sewer, 2010).
The mechanism by which $FB_1$ increases porcine granulosa cell progesterone production will require elucidation. However, considering that $FB_1$, structurally resembling sphingoid bases, inhibits ceramide synthase and thus disrupts the *de novo* biosynthesis of ceramide (Wang et al., 1991; Merrill et al., 2001), these

results are supported by studies indicating ceramide as a suppressor of progesterone biosynthesis (Santana et al., 1996; Budnik et al. 1999; Morales et al., 2003; Lucki and Sewer, 2010). In ovarian granulosa cells, activation of sphingomyelinase by IL-1β suppresses progesterone production in a ceramide-dependent manner (Santana et al., 1996). Further, Budnik et al. (1999) reported that sphingomyelin hydrolysis also resulted in decreased progesterone secretion in MA-10 mouse Leydig tumor cells by suppressing StAR protein expression. Similarly, ceramide-dependent StAR protein suppression was also reported in rat Leydig cells where it resulted in decreased testosterone synthesis (Morales et al., 2003).

Since co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is a common feature of several surveys raising the possibility of toxicological interactions, granulosa cells were also exposed to $FB_1$ in combination with DON or α-ZOL. The effects of DON and α-ZOL on steroid production by porcine granulosa cells have been previously reported (Tiemann et al., 2003; Ranzenigo et al., 2008; Medvedova et al., 2011). Consistent with the results of Ranzenigo et al. (2008), in the present study DON at 3.4 μM (1000 ng/mL) strongly inhibited FSH plus IGF-I-induced progesterone and estradiol production. On the contrary, Medvedova et al. (2011) found that DON at 3.4 μM increased progesterone release by porcine granulosa cells. However, this occurred at different incubation conditions than in the present study as granulosa cells were exposed to DON in the presence of serum and exposure that lasted 24 h. Different from porcine granulosa cells cultured in serum-free medium, cells cultured in serum-containing medium are dependent on exogenous lipoprotein cholesterol for progesterone biosynthesis (Veldhuis et al., 1984). The inhibitory effect of DON on steroid production was not affected by $FB_1$ as no significant difference was detected in combination with $FB_1$.
α-ZOL (9.4 μM) showed a stimulatory effect on progesterone production induced by FSH plus IGF-I in agreement with the study conducted by Ranzenigo et al. (2008). Combination of $FB_1$ with α-ZOL produced additive effects suggesting that these two *Fusarium* mycotoxins may act by different mechanisms of action. In addition, our findings that α-ZOL decreased estradiol production only in the presence of $FB_1$ suggests that $FB_1$ may be sensitizing granulosa cells to the inhibitory effect of α-ZOL on estradiol production.

In conclusion, results of the present study indicate that $FB_1$ has direct effects on granulosa cell steroidogenesis in swine, and are the first to evaluate the interaction between $FB_1$ and DON or α-ZOL in granulosa cells. These interactions and direct ovarian effects could be one cause of swine reproductive failures.

## 4.6. References

**Aad P.Y., Voge J.L., Santiago C.A., Malayer J.R., Spicer L.J.** (2006). Real-Time RT-PCR Quantification of pregnancy- associated plasma protein-A mRNA abundance in bovine granulosa and theca cells: effects of hormones *in vitro*. *Domestic Animal Endocrinology, 31: 357-372.*

**Alm H., Brussow K.P., Torner H., Vanselow J., Tomek W., Danicke S., Tiemann U.** (2006). Influence of *Fusarium*-toxin contaminated feed on initial quality and meiotic competence of gilt oocytes. *Reproductive Toxicology, 22: 44-50.*

**Alm H., Greising T., Brüssow K.P., Torner H., Tiemann U.** (2002). The influence of the mycotoxins deoxynivalenol and zearalenol on *in vitro* maturation of pig oocytes and *in vitro* culture of pig zygotes. *Toxicology In Vitro, 16: 643-648.*

**Bacon C.W., Porter J.K., Norred W.P.** (1995). Toxic interaction of fumonisin $B_1$ and fusaric acid measured by injection into fertile chicken egg. *Mycopathologia, 129: 29-35.*

**Baraño J.L.S., Hammond J.M.** (1985). Serum-free medium enhances growth and differentiation of cultured pig granulosa cells. *Endocrinology, 116: 51-58.*

**Baraño J.L.S., Hammond J.M.** (1986). FSH increases the synthesis and stores of cholesterol in porcine granulosa cells. *Molecular and Cellular Endocrinology, 44: 227-236.*

**Boutigny A.L., Beukes I., Small I., Zuhlke S., Spiteller M., Van Rensburg B.J., Flett B., Viljoen A.** (2012). Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. *Plant pathology, 61: 522-531.*

**Budnik L.T., Jahner D., Mukhopadhyay A.K.** (1999). Inhibitory effects of TNF alpha on mouse tumor Leydig cells: possible role of ceramide in the mechanism of action. *Molecular and Cellular Endocrinology, 150: 39-46.*

**Caloni F., Ranzenigo G., Cremonesi F., Spicer L.J.** (2009). Effects of a trichothecene, T-2 toxin, on proliferation and steroid production by porcine granulosa cells. *Toxicon, 54: 337-344.*

**EFSA (European Food Safety Authority)** (2005). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission

related to fumonisins as undesirable substances in animal feed. *EFSA Journal, 235: 1-32.*

**Ewuola E.O., Egbunike G.N.** (2010). Effects of dietary fumonisin $B_1$ on the onset of puberty, semen quality, fertility rates and testicular morphology in male rabbits. *Reproduction, 13: 439-445.*

**Floss J.L., Casteel S.W., Johnson G.C., Rottinghaus G.E., Krause G.F.** (1994). Development toxicity of fumonisin in Syrian hamsters. *Mycopathologia, 128: 33-38.*

**Flynn T.J., Pritchard D., Bradlaw J., Eppley R., Page S.** (1996). *In vitro* embryotoxicity of fumonisin $B_1$ evaluated with cultured postimplantation staged rat embryos. *In Vitro Toxicology, 9: 271-279.*

**Garrido C.E., Hernández Pezzani C., Pacin, A.** (2012). Mycotoxins occurrence in Argentina's maize (Zea mays L.), from 1999 to 2010. *Food Control, 2: 660-665.*

**Gbore F.A.** (2009a). Growth performance and puberty attainment in growing pigs fed dietary fumonisin $B_1$. *Journal of Animal Physiology and Animal Nutrition, 93: 761-767.*

**Gbore F.A.** (2009b). Reproductive organ weights and semen quality of pubertal boars fed dietary fumonisin $B_1$. *Animal, 3: 1133-1137.*

**Gbore F.A., Egbunike G.N.** (2008). Testicular and epididymal sperm reserves and sperm production of pubertal boars fed dietary fumonisin $B_1$. *Animal Reproduction Science, 105: 392-397.*

**Gbore F.A., Owolawi T.J., Erhunwunsee M., Akele O., Gabriel-Ajobiewe R.A.O.** (2012). Evaluation of the Reproductive Toxicity of Dietary Fumonisin $B_1$ in Rats. *Jordan Journal of Biological Sciences, 5: 183-190.*

**Gelderblom W.C., Kriek N.P., Marasas W.F.O., Thiel P.G.** (1991). Toxicity and carcinogenicity of the *Fusarium* moniliforme metabolite, fumonisin $B_1$, in rats. *Carcinogenesis, 12: 1247-1251.*

**Graham J.D., Clarke C.L.** (1997). Physiological action of progesterone in target tissues. *Endocrine Reviews, 18: 502-519.*

**Gumprecht L.A., Marcucci A., Weigel R.M., Vesonder R.F., Riley R.T., Showker J.L., Beasley V.R., Haschek W.M.** (1995). Effects of intravenous fumonisin $B_1$ in rabbits: nephrotoxicity and sphingolipid alterations. *Natural Toxins, 3: 395-403.*

**Harrison L.R., Colvin B.M., Greene J.T., Newman L.E., Cole J.R. Jr.** (1990). Pulmonary edema and hydrothorax in swine produced by fumonisin $B_1$, a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostic Investigation, 2: 217-221.*

**Howard P.C., Eppley R.M., Stack M.E., Warbritton A., Voss K.A., Lorentzen R.J., Kovach R.M., Bucci T.J.** (2001a). Fumonisin $B_1$ carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. *Environmental Health Perspectives, 109: 277-282.*

**Howard P.C., Warbritton A., Voss K.A., Lorentzen R.J., Thurman J.D., Kovach R.M., Bucci T.J.** (2001b). Compensatory regeneration as a mechanism for renal tubule carcinogenesis of fumonisin $B_1$ in F344/N/Nctr BR rats. *Environmental Health Perspectives, 109: 309-314.*

**Hsueh A.J.W., Adashi E.Y.P., Jones P.B.C., Welsh T.H. Jr.** (1984). Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocrine Reviews, 5: 76-127.*

**Javed T., Richard J.L., Bennett G.A., Dombrink-Kurtzman M.A., Bunte R.M., Koelkebeck K.W., Côté L.M., Leeper R.W., Buck W.B.** (1993). Embryopathic and embryocidal effects of purified fumonisin $B_1$ or *Fusarium proliferatum* culture material extract on chicken embryos. *Mycopathologia, 123: 185-193.*

**Kellerman T.S., Marasas W.F.O., Thiel P.G., Gelderblom W.C., Cawood M., Coetzer J.A.** (1990). Leukoencephalomalacia in two horses induced by oral dosing of fumonisin $B_1$. *Onderstepoort Journal of Veterinary Research, 57: 269-275.*

**Lahav M., Garmey J.C., Veldhuis J.D.** (1996). Paradoxical effect of 3-isobutyl-1-methylxanthine on cytochrome P450 cholesterol side- chain cleavage mRNA accumulation in porcine granulosa cells. *Molecular and Cellular Endocrinology, 117: 203-210.*

**Langhout D.J., Spicer L.J., Geisert R.D.** (1991). Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and

gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. *Journal of Animal Science, 69: 3321-3334.*

**Lucki N.C., Sewer M.B.** (2010). The interplay between bioactive sphingolipids and steroid hormones. *Steroids, 75: 390-399.*

**Malekinejad H., Schoevers E.J., Daemen I.J.J.M., Zijstra C., Colenbrander B., Fink-Gremmels J., Roelen B.A.J.** (2007). Exposure of oocytes to the *Fusarium* toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. *Biology of Reproduction, 77: 840-847.*

**Marin S., Magan N., Ramos A.J., Sanchis V.** (2004). Fumonisin- producing strains of *Fusarium*: a review of their ecophysiology. *Journal of Food Protection, 5: 1792-1805.*

**Medvedova M., Kolesarova A., Capcarova M., Labuda R., Sirotkin A.V., Kovacik J., Bulla J.** (2011). The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells *in vitro*. *Journal of Environmental Science and Health - Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 46: 213-219.*

**Merrill A.H. Jr., Sullards M.C., Wang E., Voss K.A., Riley R.T.** (2001). Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives, 109: 283-289.*

**Minervini F., Lacalandra G.M., Filannino A., Garbetta A., Nicassio M., Dell'Aquila M.E., Visconti A.** (2010). Toxic effects induced by mycotoxin fumonisin $B_1$ on equine spermatozoa: assessment of viability, sperm chromatin structure stability, ROS production and motility. *Toxicology In Vitro, 24: 2072-2078.*

**Morales V., Santana P., Díaz R., Tabraue C., Gallardo G., López Blanco F., Hernández I., Fanjul L.F., Ruiz de Galarreta C.M.** (2003). Intratesticular delivery of tumor necrosis factor-alpha and ceramide directly abrogates steroidogenic acute regulatory protein expression and leydig cell steroidogenesis in adult rats. *Endocrinology, 144: 4763-4772.*

**Ogunlade J.T., Ewuola E.O., Gbore F.A., Bandyopadhyay R., Niezen J., Egbunike G.N.** (2006). Testicular and epididymal sperm reserves of rabbits fed fumonisin contaminated diets. *World Applied Sciences Journal, 1: 35-38.*

**Ott L.** (1977). Multiple comparisons. In: *An Introduction to Statistical Methods and Data Analysis. Duxbury Press, North Scituate, MA, pp. 384-388.*

**Petro E.M., Leroy J.L., Van Cruchten S.J., Covaci A., Jorssen E.P., Bols P.E.** (2012). Endocrine disruptors and female fertility: focus on (bovine) ovarian follicular physiology. *Theriogenology, 78: 1887-1900.*

**Ranzenigo G., Caloni F., Cremonesi F., Aad P.Y., Spicer L.J.** (2008). Effects of *Fusarium* mycotoxins on steroid production by porcine granulosa cells. *Animal Reproduction Science, 107: 115-130.*

**Rodrigues I., Naehrer K.** (2012). A Three-Year Survey on the Worldwide Occurrence of Mycotoxins in Feedstuffs and Feed. *Toxins, 4: 663-675.*

**Santana P., Llanes L., Hernandez I., Gonzalez-Robayna I., Tabraue C., Gonzalez-Reyes J., Quintana J., Estevez F., Ruiz de Galarreta C.M., Fanjul L.F.** (1996). Interleukin-1 beta stimulates sphingomyelin hydrolysis in cultured granulosa cells: evidence for a regulatory role of ceramide on progesterone and prostaglandin biosynthesis. *Endocrinology, 137: 2480-2489.*

**SAS** (1999). The SAS System for Windows: Version 8. *SAS Institute Inc., Cary, NC.*

**Schoevers E.J., Fink-Gremmels J., Colenbrander B., Roelen B.A.** (2010). Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. *Theriogenology, 74: 968-978.*

**Spicer L.J., Kao L.C., Strauss III J.F., Hammond J.M.** (1990). 2-hydroxyestradiol enhanced progesterone production by porcine granulosa cells: dependence on *de novo* cholesterol synthesis and stimulation of cholesterol side-chain cleavage activity and cytochrome P450scc messenger ribonucleic acid levels. *Endocrinology, 127: 2763-2770.*

**Spicer L.J., Aad P.Y.** (2007). Insulin-like growth gactor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. *Biology of Reproduction, 77: 18-27.*

**Spicer L.J., Alpizar E., Echternkamp S.E.** (1993). Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation,

progesterone production, estradiol production, and(or) insulin-like growth factor I production *in vitro*. *Journal of Animal Science, 71: 1232-1241.*

**Spicer L.J., Chamberlain C.S.** (1998). Influence of cortisol on insulin- and insulin-like growth factor 1 (IGF-1)-induced steroid production and on IGF-1 receptors in cultured bovine granulosa cells and thecal cells. *Endocrine, 9: 153-161.*

**Spicer L.J., Chamberlain C.S., Maciel S.M.** (2002). Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. *Domestic Animal Endocrinology, 22: 237-254.*

**Spicer L.J., Hammond J.M.** (1987). 2-Hydroxyestradiol modulates a facilitative action of catecholamines on porcine granulosa cells. *Endocrinology, 120: 2375-2382.*

**Spicer L.J., Hammond J.M.** (1989). Catecholestrogens inhibit proliferation and DNA synthesis of porcine granulosa cells *in vitro*: comparison with estradiol, 5α-dihydrotestosterone, gonadotropins and catecholamines. *Molecular and Cellular Endocrinology, 64: 119-126.*

**Spicer L.J., Kao L.C., Strauss III J.F., Hammond J.M.** (1990). 2-Hydroxyestradiol enhanced progesterone production by porcine granulosa cells: dependence on *de novo* cholesterol synthesis and stimulation of cholesterol side-chain cleavage activity and cytochrome P450scc messenger ribonucleic acid levels. *Endocrinology, 127: 2763-2770.*

**Tiemann U., Tomek W., Schneider F., Vanselow J.** (2003). Effects of the mycotoxins α- and β-zearalenol on regulation of progesterone synthesis in cultured granulosa cells from porcine ovaries. *Reproductive Toxicology, 17: 673-681.*

**Veldhuis J.D., Gwynne J.T., Strauss III J.F., Demers L.M., Klase P.A.** (1984). Role of estradiol as a biological amplifier of gonadotropin action in the ovary: *in vitro* studies using swine granulosa cells and homologous, lipoproteins. *Endocrinology, 114: 2312-2322.*

**Voge J.L., Santiago C.A., Aad P.Y., Goad D.W., Malayer J.R., Spicer L.J.** (2004). Effect of insulin like growth factors (IGF), FSH, and leptin on IGF-binding-protein mRNA expression in bovine granulosa and theca cells: quantitative detection by real-time PCR. *Peptides, 25: 2195-2203.*

**Voss K.A., Chamberlain W.J., Bacon C.W., Norred W.P.** (1993). A preliminary investigation on renal and hepatic toxicity in rats fed purified fumonisin $B_1$. *Natural Toxins, 1: 222-228.*

**Wang E., Norred W.P., Bacon C.W., Riley R.T., Merrill A.H. Jr.** (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *The Journal of Biological Chemistry, 266: 14486-14490.*

**Zhu L., Yuan H., Guo C., Lu Y., Deng S., Yang Y., Wei Q., Wen L., He Z.** (2012). Zearalenone induces apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9-dependent mitochondrial signaling pathway. *Journal of Cellular Physiology, 227: 1814-1820.*

# CHAPTER 5

# General Discussion

# 5. General discussion

On a global scale, cereal grains and animal feed may be subject to multiple contamination with trichothecenes, zearalenone (ZEA) and fumonisins, the major mycotoxins of *Fusarium* fungi (Placinta et al., 1999). Contamination of feed grains is inevitable since many toxigenic species of *Fusarium* are also common phytopathogens, producing cereal crop diseases which are difficult to control (D'Mello, 1999). Giving contaminated feeds to susceptible animals poses a serious threat to animal health and productivity and cause great economic losses, by acting directly or indirectly on fertility (Gbore et al., 2012). Reproductive inefficiency is recognized as the most costly limiting constraint to efficient animal production as reproduction is the bedrock of animal production (Gbore, 2009a; Ewuola and Egbunike, 2010). This makes the assessment of the effects of *Fusarium* mycotoxins on animal reproduction crucial in an effort to improve livestock production (Gbore et al., 2012).
There is now unequivocal evidence implicating ZEA in reproductive disorders of swine and other domestic animals. Experiments *in vivo* and *in vitro* indicate that ZEA and its metabolites exert estrogenic effects resulting in functional and morphological alteration in reproductive organs. Hence, ZEA is grouped with the compounds known collectively as endocrine disrupters (Fink-Gremmels and Malekinejad, 2007). Recently the potential of trichothecenes to act as endocrine disruptors has been investigated. Deoxynivalenol (DON) was found to inhibit *in vitro* porcine oocyte maturation (Alm et al., 2002, 2006; Malekinejad et al., 2007; Schoevers et al., 2010) and to affect steroid production and proliferation of cultured porcine granulosa cells (Ranzenigo et al., 2008; Medvedova et al., 2011). Similar to DON, T-2 toxin showed potent dose-dependent effects on pig granulosa cell proliferation and steroidogenesis (Caloni et al., 2009). Moreover, animal studies have shown abnormal reproductive effects of T-2 toxin (Huszenicza et al., 2000). So far very little information is available on the potential reproductive toxicity of fumonisins. Fumonisin $B_1$ ($FB_1$) is the most significant of the fumonisins in terms of toxicity and occurrence (EFSA, 2005) and recent surveys have raised concerns about the widespread occurrence of this mycotoxin in corn and corn-based products and its implication for animal health and productivity (Boutigny et al., 2012; Garrido et al., 2012; Rodrigues and Naehrer, 2012). Animal studies reported a delay attainment of sexual maturity and a significant reduction of sperm production and semen quality in pigs (Gbore and Egbunikem, 2008; Gbore, 2009a; Gbore, 2009b) and rabbits (Ewuola and Egbunike, 2010) after dietary exposure to $FB_1$. Moreover, $FB_1$ was found to affect *in vitro* some functional parameters of equine spermatozoa, such as sperm chromatin stability and motility, with potential to determine subfertility

in stallion (Minervini et al., 2010). Until now, no study has evaluated direct effects of $FB_1$ on the ovary. Considering that pigs are high consumers of corn and are thus particularly exposed to fumonisins (Colvin and Harrison, 1992), in the present thesis primary porcine granulosa cells were used to determine the effect of $FB_1$ on pig reproductive functions. Granulosa cells are the maximal cell population in follicles, and they play an essential role in the process of normal folliculogenesis and oocyte growth and development (Petro et al., 2012). Therefore, the aim of the present thesis was to evaluate *in vitro* if $FB_1$ can impair swine reproductive function *via* affecting granulosa cell proliferation (chapter 3), steroidogenesis and gene expression (chapter 4). Because co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is an alarming feature of several surveys, the interaction between $FB_1$ and DON or α-zearalenol (α-ZOL), ZEA major active metabolite, was also investigated.

The results achieved indicate that $FB_1$ has direct and potent effects on granulosa cell proliferation and steroidogenesis. In the first study (chapter 3), $FB_1$ was found to inhibit granulosa cell proliferation in accordance with results previously obtained with porcine epithelial cell lines of renal (LLC-PK1) and intestinal (IPEC-1) origins (Yoo et al., 1992; Bouhet et al., 2004) and primary porcine lymphocytes (Marin et al., 2007). The precise mechanism by which $FB_1$ inhibits porcine granulosa cell proliferation will need in-depth studies but based on the previous studies (Yoo et al., 1992; Yoo et al., 1996; Riley et al., 1999) conducted with LLC-PK1, a cell line, which is considered to be a good model for studying $FB_1$ *in vitro* effects, there is a close correlation between fumonisin-induced disruption of sphingolipid metabolism and inhibition of cell growth. $FB_1$, structurally resembling sphingoid bases, inhibits ceramide synthase and thus disrupts the *de novo* biosynthesis of ceramide and sphingolipid metabolism (Wang et al., 1991; Merrill et al., 2001; Voss et al., 2007). The inhibition of ceramide synthase leads to the accumulation of sphingoid bases (sphinganine and, to a lesser degree, sphingosine) which are known to mediate several key biological processes such as cell proliferation or DNA replication (Spiegel and Merrill, 1996). Moreover, results of the present study revealed that DON was much more potent than $FB_1$ at inhibiting cell growth and no significant difference was detected in combination with $FB_1$. α-ZOL (9.4 μM) showed a stimulatory effect on granulosa cell numbers even in combination with $FB_1$. Granulosa cells provide essential nutrients to the oocyte and establish a link between the oocyte and the surrounding ovarian tissue in which the follicle is embedded (Petro et al., 2012). Therefore, the ability of these mycotoxins to alter granulosa cell proliferation may compromise the normal follicle growth and oocyte survival.

Granulosa cells are not only responsible for the delivery of nutrients to the oocyte but they also attribute to the ovarian steroidogenesis (Petro et al., 2012). In the second study (chapter 4), $FB_1$ was found to increase granulosa cell

progesterone production induced by FSH and IGF-I. Progesterone is an essential regulator of the reproductive events and plays key roles in ovulation, zygote implantation and the subsequent maintenance of pregnancy (Graham and Clarke, 1997). In these experiments $FB_1$ was found to decrease the expression of the mitochondrial enzyme cytochrome P450scc (CYP11A1), which catalyzes the conversion of cholesterol to pregnenolone. Moreover, the stimulatory effect of $FB_1$ on progesterone production was found to be not dependent on *de novo* cholesterol synthesis. Therefore, two different mechanisms for $FB_1$-induced progesterone synthesis may be hypothesised: (i) increased activity of the microsomal enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD), which catalyzed the conversion of pregnenolone to progesterone, and/or (ii) increased expression of ovarian steroidogenic acute regulatory (StAR) protein, which mediates the transfer of cholesterol to the inner mitochondrial membrane, where P450scc resides. The mechanism by which $FB_1$ increases porcine granulosa cell progesterone production will require elucidation. However, considering that $FB_1$ disrupts the *de novo* biosynthesis of ceramide (Wang et al., 1991; Merrill et al., 2001), these results are supported by works indicating ceramide as a suppressor of progesterone biosynthesis (Santana et al., 1996; Budnik et al. 1999; Morales et al., 2003; Lucki and Sewer, 2010). Moreover, in the present study, DON was found to strongly inhibit FSH plus IGF-I-induced steroid production and $FB_1$ had no significant effect on the DON response. α-ZOL showed a stimulatory effect on progesterone production and, interestingly, combination of $FB_1$ with α-ZOL produced additive effects suggesting that these two *Fusarium* mycotoxins may act by different mechanisms of action. In addition, our findings that α-ZOL decreased estradiol production only in the presence of $FB_1$ suggests that $FB_1$ may be sensitizing granulosa cells to the inhibitory effect of α-ZOL on estradiol production. These experiments show that $FB_1$ as well as DON and α-ZOL can influence the steroidogenic capacity of the granulosa cells, thereby demonstrating the ability of these *Fusarium* mycotoxins to disturb the critical well-balanced endocrine regulation of the developing follicle, which is essential to reach the pre-ovulatory stage (Petro et al., 2012).

In conclusion, results of the present thesis indicate that $FB_1$ may be able to alter growth of the granulosa cell layer within ovarian follicles in addition to its effect on steroid production and provide the first information on the interactions between $FB_1$ and DON or α-ZOL in granulosa cells. The direct ovarian effects of these *Fusarium* mycotoxins and their interaction could be one mechanism whereby contaminated feed could impair swine reproductive function.

## 5.1. References

**Alm H., Brussow K.P., Torner H., Vanselow J., Tomek W., Danicke S., Tiemann U.** (2006). Influence of *Fusarium*-toxin contaminated feed on initial quality and meiotic competence of gilt oocytes. *Reproductive Toxicology, 22: 44-50.*

**Alm H., Greising T., Brüssow K.P., Torner H., Tiemann U.** (2002). The influence of the mycotoxins deoxynivalenol and zearalenol on *in vitro* maturation of pig oocytes and *in vitro* culture of pig zygotes. *Toxicology In Vitro, 16: 643-648.*

**Bouhet S., Hourcade E., Loiseau N., Fikry A., Martinez S., Roselli M., Galtier P., Mengheri E., Oswald I.P.** (2004). The mycotoxin fumonisin $B_1$ alters the proliferation and the barrier function of porcine intestinal epithelial cells. *Toxicological Sciences, 77: 165-171.*

**Budnik L.T., Jahner D., Mukhopadhyay A.K.** (1999). Inhibitory effects of TNF alpha on mouse tumor Leydig cells: possible role of ceramide in the mechanism of action. *Molecular and Cellular Endocrinology, 150: 39-46.*

**Caloni F., Ranzenigo G., Cremonesi F., Spicer L.J.** (2009). Effects of a trichothecene, T-2 toxin, on proliferation and steroid production by porcine granulosa cells. *Toxicon, 54: 337-344.*

**Colvin B.M., Harrison L.R.** (1992). Fumonisin-induced pulmonary edema and hydrothorax in swine. *Mycopathologia, 117: 79-82.*

**Creppy E.E.** (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127: 19-28.*

**D'Mello J.P.F., Placinta C.M., Macdonald A.M.C.** (1999). *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology, 80: 183-205.*

**EFSA (European Food Safety Authority)** (2005). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to fumonisins as undesirable substances in animal feed. *EFSA Journal, 235: 1-32.*

**Fink-Gremmels J., Malekinejad H.** (2007). Biochemical mechanisms and clinical effects associated with exposure to the mycoestrogen zearalenone. *Animal Feed Science and Technology, 137: 326-341.*

**Gbore F.A.** (2009a). Growth performance and puberty attainment in growing pigs fed dietary fumonisin $B_1$. *Journal of Animal Physiology and Animal Nutrition, 93: 761-767.*

**Gbore F.A.** (2009b). Reproductive organ weights and semen quality of pubertal boars fed dietary fumonisin $B_1$. *Animal, 3: 1133-1137.*

**Gbore F.A., Egbunike G.N.** (2008). Testicular and epididymal sperm reserves and sperm production of pubertal boars fed dietary fumonisin $B_1$. *Animal Reproduction Science, 105: 392-397.*

**Gbore F.A., Owolawi T.J., Erhunwunsee M., Akele O., Gabriel-Ajobiewe R.A.O.** (2012). Evaluation of the Reproductive Toxicity of Dietary Fumonisin $B_1$ in Rats. *Jordan Journal of Biological Sciences, 5: 183-190.*

**Huszenicza G., Fekete S., Szigeti G., Kulcsár M., Fébel H., Kellems R.O., Nagy P., Cseh S., Veresegyházy T., Hullár I.** (2000). Ovarian consequences of low dose peroral *Fusarium* (T-2) toxin in a ewe and heifer model. *Theriogenology, 53: 1631-1639.*

**Lucki N.C., Sewer M.B.** (2010). The interplay between bioactive sphingolipids and steroid hormones. *Steroids, 75: 390-399.*

**Malekinejad H., Schoevers E.J., Daemen I.J.J.M., Zijstra C., Colenbrander B., Fink-Gremmels J., Roelen B.A.J.** (2007). Exposure of oocytes to the *Fusarium* toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. *Biology of Reproduction, 77: 840-847.*

**Marin D.E., Gouze M.E., Taranu I., Oswald I.P.** (2007). Fumonisin $B_1$ alters cell cycle progression and interleukin-2 synthesis in swine peripheral blood mononuclear cells. *Molecular Nutrition and Food Research, 51: 1406-1412.*

**Medvedova M., Kolesarova A., Capcarova M., Labuda R., Sirotkin A.V., Kovacik J., Bulla J.** (2011). The effect of deoxynivalenol on the secretion activity, proliferation and apoptosis of porcine ovarian granulosa cells *in vitro*. *Journal of Environmental Science and Health - Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 46: 213-219.*

**Merrill A.H. Jr., Sullards M.C., Wang E., Voss K.A., Riley R.T.** (2001). Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environmental Health Perspectives, 109: 283-289.*

**Minervini F., Lacalandra G.M., Filannino A., Garbetta A., Nicassio M., Dell'Aquila M.E., Visconti A.** (2010). Toxic effects induced by mycotoxin fumonisin $B_1$ on equine spermatozoa: assessment of viability, sperm chromatin structure stability, ROS production and motility. *Toxicology In Vitro, 24: 2072-2078.*

**Morales V., Santana P., Díaz R., Tabraue C., Gallardo G., López Blanco F., Hernández I., Fanjul L.F., Ruiz de Galarreta C.M.** (2003). Intratesticular delivery of tumor necrosis factor-alpha and ceramide directly abrogates steroidogenic acute regulatory protein expression and leydig cell steroidogenesis in adult rats. *Endocrinology, 144: 4763-4772.*

**Petro E.M., Leroy J.L., Van Cruchten S.J., Covaci A., Jorssen E.P., Bols P.E.** (2012). Endocrine disruptors and female fertility: focus on (bovine) ovarian follicular physiology. *Theriogenology, 78: 1887-1900.*

**Placinta C.M., D'Mello C.P.F., MacDonald A.M.C.** (1999). A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Animal Feed Science and Technology, 78: 21-37.*

**Ranzenigo G., Caloni F., Cremonesi F., Aad P.Y., Spicer L.J.** (2008). Effects of *Fusarium* mycotoxins on steroid production by porcine granulosa cells. *Animal Reproduction Science, 107: 115-130.*

**Riley R.T., Voss K.A., Norred W.P., Bacon C.W., Meredith F.I., Sharma R.P.** (1999). Serine palmitoyltransferase inhibition reverses anti-proliferative effects of ceramide synthase inhibition in cultured renal cells and suppresses free sphingoid base accumulation in kidney of BALBc mice. *Environmental Toxicology and Pharmacology, 7: 109-118.*

**Santana P., Llanes L., Hernandez I., Gonzalez-Robayna I., Tabraue C., Gonzalez-Reyes J., Quintana J., Estevez F., Ruiz de Galarreta C.M., Fanjul L.F.** (1996). Interleukin-1 beta stimulates sphingomyelin hydrolysis in cultured granulosa cells: evidence for a regulatory role of ceramide on progesterone and prostaglandin biosynthesis. *Endocrinology, 137: 2480-2489.*

**Schoevers E.J., Fink-Gremmels J., Colenbrander B., Roelen B.A.** (2010). Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. *Theriogenology, 74: 968-978.*

**Spiegel S., Merrill A.H. Jr.** (1996). Sphingolipid metabolism and cell growth regulation. *The FASEB journal, 10: 1388-1397.*

**Voss K.A., Smith G.W., Haschek W.M.** (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. *Animal Feed Science and Technology, 137: 299-325.*

**Wang E., Norred W.P., Bacon C.W., Riley R.T., Merrill A.H. Jr.** (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme. The Journal of Biological Chemistry, 266: 14486-14490.*

**Yoo H.S., Norred W.P., Showker J., Riley R.T.** (1996). Elevated sphingoid bases and complex sphingolipid depletion as contributing factors in fumonisin-induced cytotoxicity. *Toxicology and Applied Pharmacology, 138: 211-218.*

**Yoo H.S., Norred W.P., Wang E., Merrill A.H. Jr., Riley R.T.** (1992). Fumonisin inhibition of *de novo* sphingolipid biosynthesis and cytotoxicity are correlated in LLC-PK1 cells. *Toxicology and Applied Pharmacology, 114: 9-15.*

# CHAPTER 6

# Summary

# 6. Summary

Fumonisins, zearalenone (ZEA) and trichothecenes, such as deoxynivalenol (DON) and T-2 toxin, are the major *Fusarium* mycotoxins occurring throughout the world in cereal grains and animal feeds. Exposure to these mycotoxins has been positively linked to reproductive disorders in pigs. However, direct ovarian effects of fumonisin $B_1$ ($FB_1$) and its interaction with DON or α-zearalenol (α-ZOL), zearalenone major active metabolite, have so far not been investigated. Thus, the goal of this thesis was to determine if $FB_1$, alone or combined with DON or α-ZOL, can impair swine reproductive activity *via* affecting granulosa cell function. To this aim, two different studies were designed.

In the first study the effects of $FB_1$ alone and combined with DON or α-ZOL on granulosa cell proliferation were evaluated. Porcine granulosa cells from small ovarian follicles (1-5 mm) were cultured for 2 days in 5% fetal bovine serum and 5% porcine serum-containing medium followed by 2 days in serum-free medium containing $FB_1$ at various doses (0, 0.01, 0.4, 10 and 14 μM) and combinations. At the end of the experiments, numbers of granulosa cells were determined using a Coulter counter. The results revealed that $FB_1$ had inhibitory effects on granulosa cell proliferation at doses ≥ 10 μM. DON (3.4 μM) strongly inhibited (by 80%; $P < 0.0001$) granulosa cell proliferation and no significant difference was detected in combination with $FB_1$. α-ZOL (9.4 μM) showed a stimulatory effect ($P < 0.01$) on granulosa cell numbers, even when treated in combination with $FB_1$.

In the second study the effects of $FB_1$ alone and combined with DON or α-ZOL on granulosa cell steroid production and gene expression were investigated. Porcine granulosa cells from small follicles (1-5 mm) were cultured as described above. At the end of the experiments, concentrations of progesterone and estradiol in culture medium were determined by radioimmunoassays. For RNA experiments, granulosa cells were lysed, RNA extracted and RNA quantity determined spectrophotometrically. Real-time RT-PCR was used to elucidate the effects of $FB_1$ on gene expression of P450scc (CYP11A1) and aromatase (CYP19A1). All doses of $FB_1$ (i.e., 0.01, 0.4, 10 and 14 μM) had no significant effect on estradiol production, whereas $FB_1$ showed a stimulatory effect on progesterone production induced by FSH plus insulin-like growth factor-I (IGF-I) at 10 and 14 μM. α-ZOL (9.4 μM) increased ($P < 0.0001$) FSH plus IGF-I-induced progesterone production by 51%. Combination of $FB_1$ with α-ZOL resulted in an increase of progesterone production (91%; $P < 0.0001$) that was significantly higher than that induced by either *Fusarium* mycotoxin alone. DON drastically inhibited (by 74%; $P < 0.0001$) progesterone production and $FB_1$ had little effect on this response. α-ZOL had no effect on FSH plus IGF-I-induced estradiol production, whereas decreased ($P < 0.05$)

estradiol production when co-treated with $FB_1$. DON (3.4 μM) strongly inhibited (by 67%; $P < 0.0001$) estradiol production and no difference was detected in combination with $FB_1$ (10 μM). $FB_1$ (10 μM) had no effect on granulosa cell CYP19A1 mRNA abundance, whereas it decreased (by 23%; $P < 0.0001$) granulosa cell CYP11A1 mRNA abundance induced by FSH plus IGF-I. In conclusion, the present thesis indicates that $FB_1$ has direct effects on porcine granulosa cell proliferation, steroid production and gene expression and provides information on the interactions between $FB_1$ and DON or α-ZOL in granulosa cells. These interactions and direct ovarian effects should be considered in swine reproductive failures.

# CHAPTER 7

# Acknowledgements

# 7. Acknowledgements

Foremost, I would like to thank my supervisor, Professor Francesca Caloni for her encouragement, support, enthusiasm, constant inspiration, innovative ideas, perspicacious thoughts, and sound advice. Her incessant guidance helped me throughout my Ph.D. years.
My thanks also go out to the support I received from the collaborative work undertaken with the Department of Animal Science, Oklahoma State University. I am especially very grateful to Professor Leon J. Spicer for his patience, motivation, enthusiasm, constructive and practical advice, and his great efforts to explain things clearly and simply.